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Do we really need management to preserve pioneer stages in wet dune slacks?

Colofon
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Chapter 1

General Introduction

Rohani Shahrudin

Dune slacks

Coastal dune slacks occur in low-lying areas between large sand-dune ridges. They are flooded during the winter and most of the spring. There are two types of dune slacks, categorized by their way of formation: **primary dune slacks** and **secondary dune slacks**. Primary dune slacks arise when sandy plains become isolated from the influence of the sea by the formation of new dune systems. They are generally very large and, if the initial lime content of the soil was high, may sustain alkaline conditions for a long time. Secondary dune slacks are formed further away from the coast under influence of the wind, generally in dunes that have not been stabilized by vegetation; they are generally smaller and tend to have a relatively short life span of several decades or less.

Young dune soils, in particular, are calcareous, because the original beach sand contains many shell fragments. This alkaline environment normally sustains a habitat with a high pH (pH>6.0). In some slacks the alkalinity is sustained by discharge of calcareous groundwater. Other characteristics of young dune slacks are low organic material content and high water tables. This kind of environment may seem quite ordinary, but dune slacks can harbour many rare and endangered plant species (Grootjans et al., 1991; Lammerts et al., 1999) and thus have attracted special attention regarding their conservation status.

Many typical of dune slack species are now threatened in NW-Europe or they have become extinct due to severe drainage, acidification and eutrophication (Van Dijk & Grootjans,1993; Davy et al., 2003). These factors affect rare dune-slack species by accelerating the succession of dune slacks; pioneer-stage communities are replaced with shrub and tree species over a very short period of time. The decline of these wet plant communities in dune slacks is thought to be caused by both natural processes and anthropogenic factors (Grootjans et al., 1991; Van Dijk & Grootjans, 1993), and this has triggered numerous activities to restore species-rich stages of dune slacks. These restoration activities offer excellent opportunities to study the impact of different management strategies on the vegetation succession and population structure of several endangered species, such as *Liparis loeselii, Pedicularis palustris, Schoenus nigricans, Dactylorhiza incarnata* and *Gentianella amarella*.

Dune-slack vegetation

The vegetation of wet dune slacks is characterized by high species diversity and low productivity. The characteristic pioneer vegetation of wet dune slacks consists mainly of plant communities that belong to the associations *Parnassio-Juncetum* and *Centaurio-Saginetum*. Aging slacks belong to the association *Junco baltici-Schoenatum nigricantis.* Mature slacks have various types of vegetation that may range from wet heathland vegetation to dune woodland vegetation. Initially, small pioneer species establish on almost bare soil, which is usually covered with a thin layer of green algae and laminated microbial mats (Grootjans et al., 1997). At the next stage, phanerogams start to colonize and adapt to the increasing nutrient availability. Most typical dune-slack species establish after the development of a moss layer. It is at this stage that red list species are the most abundant. Orchids only occur in these early successional stages, where they receive nutrients from soil mycorrhizas (Smith, 1966). Juvenile orchids in particular are almost totally dependent on nutrients transferred from mycorrhizas and they are also probably more sensitive to changes in the environment. The shift from pioneer to more mature stages usually takes around 20-30 years (Van der Maarel et al., 1985). The rate of vegetation succession in dune slacks is largely controlled by the productivity of the ecosystem,

the decomposition rate of organic matter and the recycling of nutrients within the ecosystem (Olff et al., 1993).

Key ecological factors limiting populations

Among the main ecological factors in dune slacks that affect the occurrence of these endangered species are organic accumulation, pH, and water level (Grootjans et al., 2002).

Organic matter accumulation

During early succession the amount of organic matter and nitrogen in the soil increases due to the production of litter and dead roots (Berendse, 1990). The total amount of organic matter may, therefore, be a good indicator of nutrient status in dune slacks. The same applies to the accumulation speed of organic matter as an indicator of the rate of nutrient accumulation and of the expected life span of pioneer vegetation. Accumulation of organic matter increases the nitrogen pool, which in turn can accelerate vegetation succession. In a heathland ecosystem, mineralization rate was shown to increase with increasing amount of organic matter (Berendse et al., 1998). Concomitantly, soil moisture increased, whereas soil pH declined sharply. Such changes in soil features can be expected to have an important impact upon the establishment and competitive ability of plant species and ultimately upon the dynamics of the species composition of the plant community. The rate of organic matter accumulation, however, appears to be influenced by the hydrological regime in a dune slack (Sival & Grootjans, 1996). The accumulation of organic material provides an increase in available nutrients and stimulates the growth of strong competitors like shrub and tree species.

pH

A crucial role is played by pH in maintaining the viability of dune-slack pioneer species. Most dune slack soils with basiphilous plant communities have pH(H_2O) values above 6.5. Buffering of pH above 6.5 may result from flooding and inundation with brackish surface water, dissolution of $CaCO_3$ and exchange of base cations. Certain species can only be found under very alkaline conditions. For example, the distribution pattern of orchid species *Dactylorhiza incarnata* seems to be determined entirely by an interaction between ammonium concentrations and soil pH (Dijk & Grootjans, 1998). Ammonium and pH values below 6 were experimentally shown to impede the growth of this species, whereas ammonium toxicity decreased with higher pH (Dijk & Eck, 1995). Soil acidification may negatively affect dune-slack species by reducing the germination of many seeds, thereby decreasing recruitment at the juvenile stage, but also by decreasing the vitality of adult plants (Sival, 1996).

Water level

Water table levels are important in several ways for wetland ecosystems. High water tables, for instance, can retard competitors of pioneer species such as *Calamagrostis epigejos, Salix repens* and trees. Additionally, it has been suggested that high water tables (just above ground level) during late spring are required to ensure germination of *Schoenus nigricans (*Ernst et al., 1988). Hydrology, as well as patch age, was found to be correlated to the recruitment and survivorship of species like *Liparis loeselii* (Jones, 1998). Fluctuations in the water table are strongly related to spatial and temporal variations in weather and topography (Moreno-Casasola & Vázquez, 1999). Variability in rainfall affects both the water table level and the balance between precipitation input into the system and groundwater. When flooded, plants are subject to anaerobic conditions, affecting their survival and growth. Inundation can cause local extinction of some non-wetland species and facilitate the

establishment of others. Thus, community composition will depend on the differential tolerance of plants to environmental conditions associated with inundation, particularly anoxia.

Meta-population dynamics

Many species exist in unevenly distributed or patchy habitats in the natural environment. These spatially segregated patches of suitable habitat can be connected with each other through varying degrees of dispersal. Interactions between populations of species on these patches, such as local extinction, colonization, and migration, can be defined as meta-population dynamics (Hanski, 1999). The concept of meta-populations was first introduced by Levins (1969) and it has been applied to a wide spectrum of organisms (Opdam 1991, Hanski 1993, Leinsham and Jamieson 2002, Lafferty et al. 1999, Jacquemyn et al. 2006). Many studies have been conducted to test the effects of frequent extinction and colonization processes on meta-population viability. However, most of the studies concentrated on modelling techniques, since it is quite challenging to observe the effects of extinction-colonization processes in real systems due to time constraints. Therefore, a very dynamic landscape, where extinction and colonization occur frequently, offers a great opportunity for researchers to study the effects of meta-population dynamics.

Management

Different coastal management programs have had a great impact on dune-slack vegetation. Due to past management policies that emphasized coastal defence, under which dunes were fixed and no formation of new dunes and dune slacks occurred, practically all pioneer stages have disappeared and most dune areas are covered by tall grasses and shrubs. As a consequence, nature management organizations initiated numerous restoration projects involving the creation of bare substrates to reset the succession process (Grootjans et al. 2002). Currently, the Dutch government has chosen to allow for more natural dynamics in certain nature reserves with no human settlement. In some of these areas, the regular maintenance of sea defence structures (sand dikes) has been discontinued and, in other areas, small holes in these structures have been made to permit re-flooding of degraded dune slacks and beach plains, thus creating new opportunities for colonization by endangered dune-slack species. This research study offers an opportunity to clarify the impact of different management strategies on the conservation and maintenance of endangered dune-slack species.

The selected study species

This study focuses on three species: *Liparis loeselii* (Orchidaceae)*, Pedicularis palustris* (Scrophulariaceae) and *Schoenus nigricans* (Cyperaceae)*.* All three species are typical of the pioneer stages of dune slacks. They are found under lowly productive and alkaline conditions. The occurrence of these three species on dune slacks has been monitored. The different life spans and dispersal mechanisms of these species make comparisons between them interesting.

Liparis loeselii is a small, inconspicuous, endangered orchid occurring in Europe and north-east America. *L. loeselii* is a perennial, short-lived species. Its fleshy green pseudobulb survives the winter just beneath the substrate surface. Often the previous year's pseudobulb is still present in spring, fused to the current year's, from which a new shoot emerges in late May or early June. Flowering occurs from late June to mid-July. The seeds of *L. loeselii* are minute and, because this species is often found in coastal environments, dispersal occurs by wind. Even though *L. loeselii* is known to be autogamous, it shows a higher level of genetic variation than

expected (Pillon et al., 2007). The species is listed as endangered under both the Habitat Directive and the Bern Convention, and therefore has the highest conservation priority.

Earlier research conducted in Great Britain has shown that the populations of *L. loeselii* can rapidly expand or disappear in response to changing conditions (Jones 1998; Wheeler et al. 1998).

Pedicularis palustris is limited to the northern hemisphere and occurs throughout Europe in natural fens and in moderately grazed or mown fen meadows.

This hemi-parasitic species has a biennial life cycle and reproduces exclusively by seed. Seeds are buoyant and can be dispersed by water. Only a minor fraction of seeds germinates immediately after primary dispersal in summer. During the first growing season, a rosette develops, which forms a winter bud in autumn (Ter Borg 1985). Flowering takes place in June and July of the second growing season. Flowers are pollinated by bumble bees. The species is short-lived and possesses only a short-term persistent seed bank (Thompson et al. 1997), thus making the population persistence of this species dependent on regular reproduction by seed.

Schoenus nigricans is usually found in acidic and alkaline peatlands, mineral soils rich in organic matter and calcareous marls.

Generally, the habitats of *S. nigricans* are waterlogged, with the water table slightly below the surface. The genus *Schoenus* has a large distribution in Europe, ranging

from the Scandinavian Peninsula (Hendrèn 1997) to southern Europe and Northern Africa. *S. nigricans* is wind-pollinated as a rule, but self-pollination may occur. Seeds germinate within the inflorescence if the flowering stem is broken and the inflorescence is close to the ground. Seeds are dispersed mainly by wind. Some of the seeds are held in the inflorescence, firmly in a spikelet, for most of the winter, and are subsequently released by strong winds. Seeds can be found in the flowering heads the following spring. Pollen is shed from early July onwards and seed set is in August. This species is characterized by high concentrations of silicon (Ernst et al., 1995) and is considered a typical pioneer species, important in wet dune slacks (Ernst et al., 1988; Ernst et al., 1996). The age of *S. nigricans* plants can be estimated by measuring the tussock diameter and number of inflorescences from sites with a known history. This method can be used because the population structure of *S. nigricans* for older established sites have been found to be dominated by older, larger tussocks compared to younger sites, which are characterized by greater numbers of seedlings and small-sized tussocks (Ernst et al., 1988). **Thesis Outline**

The major aim of this thesis is to compare the ecology of pioneer species during natural successional processes to that after management measures have been undertaken in order to understand the effectiveness of those management measures in prolonging the persistence of pioneer species of wet dune slacks. This thesis is comprised of work undertaken in both field and laboratory conditions, and seed germination experiments. In the following chapters, the scale of the studies decreases from ecosystem/community level **(Chapter 2, Chapter 3)**, population level **(Chapter 4)**, species level **(Chapter 5)** to finally, the sub-genus level (genetics) **(Chapter 6).**

Chapter 1 gives the background of the research and introduces the main scientific research questions of the study. **Chapter 2** deals with factors that influence the rate of organic matter accumulation, which is an important parameter to understand with respect to successional rate. We used several soil chronosequences to study soil and vegetation development. A soil chronosequence consist of soils of different age, situated close together, which makes comparisons possible over relatively long time intervals. We also used data from long-term monitoring programs (16-77 years) on the Dutch Wadden Sea islands. Using these data direct comparisons over time were possible. Field measurements consisted of pH, soil organic matter (SOM), above-ground standing crop and water levels. Water-level regimes (inundation duration and mean minimum water level) were simulated using a hydrological model. The questions were: (i) Which factors regulate accumulation rates of SOM in wet dune slacks? (ii) How long can pioneer species, such as *Littorella uniflora*, retard the accumulation of organic matter in dune slacks?

Chapter 3 – This chapter deals with the possible occurrence of alternative stable states (ASS) in wet dune slacks. One of the chronosequences that was studied in Chapter 2 (Koegelwieck, Terschelling) was studied in more detail for the purpose of analyzing the stability of different successional stages: pioneer stage, mid-successional stage and mature stage. The questions were: (i) Do alternative stable states in dune slacks really occur or are successional stages always in transition? (ii) Which successional stages are most stable and why? Several sites located in one dune slack had been sod-cut in different years and provided a great opportunity to study changes in species composition and environmental parameters (ecosystem development) during succession.

Chapter 4 – This chapter deals with the population structure of two typical dune-slack species (*Schoenus nigricans* and *Pedicularis palustris*) that occur at early stages of dune-slack development. Their population structures were studied in relation to different management practices and different environmental conditions (pH, organic matter and wetness). Questions we sought to answer were: (i) Do management activities affect the population structure and also population viability? (ii) How do populations differ in the soil pH and organic matter of their environment? (iii) Does prolonged flooding prevent germination in *P. palustris* populations? The population structure was described by distinguishing between juveniles and adults, and noting whether these adults flowered and produced ripe seed. The vitality of the seeds was assessed by seed germination tests. To test the influence of flooding on seed germination, we carried out a field experiment, in which viable seeds were sown under different flooding conditions. The results of this experiment are also reported in Chapter 4.

Chapter 5 – This chapter investigates the survival of a typical dune slack species, the orchid *Liparis loeselii*. This orchid is highly endangered in most European countries but still occurs on the Dutch Wadden Sea islands, sometimes in high numbers. This chapter deals with the performance of these orchid populations over the course of vegetation succession at a newly formed beach on the Dutch Wadden Sea island of Texel. Once again, the chronosequence approach was used to depict the environmental and demographic conditions over the entire life-span of *L. loeselii* populations. In this chapter, we had two questions: i) What is the length of the window of opportunity for *L. loeselii* on the southern tip of Texel? ii) Which factors best predict the occurrence of *L. loeselii*?

Chapter 6 – This chapter further studies the orchid *L. loeselii* but on the level of individuals within a population. We studied the effect of landscape dynamics on the genetic structure of the *L. loeselii* metapopulation of the Wadden Sea islands. We collected samples from almost all known populations of the Dutch and German Wadden Sea islands (Texel, Vlieland, Terschelling, Ameland, Schiermonnikoog and Borkum). Leaf samples from 596 individuals were collected from 28 populations. The following scientific questions were addressed: i) Is there a high level of genetic differentiation due to limited pollen dispersal? ii) Is there evidence of long-distance seed dispersal within the *L. loeselii* meta-population? iii) What is the origin of the individuals found on newly-colonized dune slacks? iv) Are older and larger populations genetically more diverse? v) Is there a correlation between genetic distance and geographical distance? For genotyping we analyzed the DNA of individual species using Amplified Fragment Length Polymorphisms (AFLP) techniques. To trace the source of colonization, and also to estimate seed dispersal distances, we used assignment tests.

Chapter 7 – The final chapter in this thesis briefly summarizes all the results to find answers to the main research questions. The effect of management decisions on the persistence of pioneer species is evaluated indirectly from a synthesis of the results. Recommendations for future research and management guidelines are also discussed in this chapter.

Chapter 2

Accumulation rates of soil organic matter in wet dune slacks on the Dutch Wadden Sea islands

Shahrudin Rohani, Bikila W. Dullo, Wilmer Woudwijk, Pieter de Hoop , Annemieke Kooijman and *Ab Grootjans*

Abstract

Background and aims
A long-term monitoring program (ranging from 16-77 years) on the Dutch Wadden Sea Islands provided well-documented examples of vegetation succession in wet dune slacks. We used this opportunity to study soil organic matter (SOM) accumulation in relation to vegetation succession. The aim of this paper is to identify the factors which regulate accumulation rates of SOM in wet dune slacks.

Methods
We used several soil chronosequences using data from the monitoring program together with data from a long-term research activity and more recent measurements. We used several soil chronosequences using data from the monitoring program together with data from a long-term research (up to 150 years) and more recent measurements. Field measurements included pH, soil organic matter, above ground standing crop and water levels. Water level regimes (inundation duration and mean minimum water level), were simulated using a hydrological model. Capable of simulating inundation duration and water-level fluctuations, this model used field measurements collected over more than 5 years, as well as precipitation and evapotranspiration data collected over a period of 25 years.

Results
Sampling two synchronic chronosequences showed that SOM accumulations increased linearly during the first 50-60 years and then levelled off. Sampling various diachronic chronosequences over time showed a wide variation in accumulation rates. Slacks with low productive species, such as *Littorella uniflora*, showed low accumulation rates (0.02-0.08 kg/m^2/year), and persisted even over a period of more than 90 years. In contrast, slacks dominated by high productive species, such as *Phragmites australis*, showed ten times higher accumulation rates (0.17- 0.26 kg/m^2/year) over a similar time period and comparable annual inundation periods (176-240 days). A multiple linear regression showed that variation in SOM accumulation rates was best explained by above-ground biomass of the vegetation.

Conclusions
We conclude that the rate of SOM accumulation in wet dune slacks is primarily controlled by plant above-ground biomass. Both above-ground biomass and SOM accumulation can remain very low over a long period of time when dune slacks are flooded during most of the year and plants with adaptive traits are able to maintain vegetation succession at a pioneer stage.

Keywords: *above-ground biomass, hydrology, nitrogen deposition, ROL, soil chronosequence, succession*

Introduction

Soil organic matter (SOM) is an important component in almost all terrestrial ecosystems, affecting the biological, chemical and physical properties of soils that influence plant growth. Changes in SOM accumulation alter many soil properties during primary succession (Walker and del Morel 2003). For example, a sharp decline in soil pH usually leads to a rapid increase in SOM and an increase in soil moisture (Jenny 1980, Van Breemen and Buurman 2002). These strong effects on soil properties indicate that SOM has profound influences on vegetation succession.
The accumulation rate of SOM is determined by litter production and litter decomposition rates, which are controlled by climatic factors, such as precipitation and temperature, and more local factors such as soil moisture, pH and type of vegetation (Alvarez and Lavado 1998, Dai and Huang 2006, Kirschbaum 1995, Parè et al. 2006). In general, climatic factors such as precipitation and temperature are recognized as the most influential factors (Jenny 1980, Alvarez and Lavado 1998). High temperatures usually stimulate decomposition rates, although excessively high temperatures may inhibit growth of soil organisms, and may consequently reduce decomposition rates (Dai and Huang 2006). Additionally, high precipitation that leads to high soil moisture promotes biomass production of the vegetation, which provides more litter input to the ecosystems thus leading to high accumulation of organic matter (Mukhortova 2008). Increased atmospheric nitrogen deposition that is observed in many European countries (e.g. 20-25 kg N $ha^{-1}yr^{-1}$ in the Dutch Wadden Sea islands; Sival and Strijkstra-Kalk 1999 compared to 3–8 kg N $ha^{-1}yr^{-1}$ in the Baltic states; Remke et al. 2009), also can increase accumulation rates in wetlands (Adema et al. 2002). With respect to soil moisture, complete water saturation leads to anaerobic conditions in the soil, which inhibit the activity of decomposers (Day 1982). Thus, prolonged inundation may lead to lower rates of litter decomposition. In peat lands, for instance, the continual process of litter accumulation and slow decomposition activity under saturated conditions leads to a high build-up of organic matter.

In wet dune slacks, however, such high rates of organic matter accumulation do not always occur. Adema et al. (2002) found remarkable differences in SOM accumulation between two sites located in the same 80-year-old dune slack, both of which were inundated for most of the year. The authors suggested that these differences in SOM accumulation represented alternative stable states, listing three possible positive feedback mechanisms to explain these differences: (1) sulphide toxicity from microbial mats; (2) occurrence of pioneer species with radial-oxygen-loss (ROL) capability; and (3) differences in the productivity of species. Dune slack species with ROL capability such as *Littorella uniflora* and *Schoenus nigricans* could facilitate rapid decomposition (Ernst et al. 1996, Adema et al. 2002). On the other hand, species with recalcitrant components in their tissues are known to slow down mineralization rates as has been shown in *Schoenus nigricans* (Ernst et al. 1996), species of *Sphagnum* (moss) (Van Breemen 1995, Verhoeven and Toth 1995, Scheffer et al. 2001) and those found in the Leguminosae (Palm and Sanchez 1991). Finally, it has been demonstrated that highly productive species usually have higher litter inputs into soil, which eventually leads to higher SOM accumulation rates, compared to species with low productivity (Smith et al. 2008).

Studying SOM accumulation rates ideally requires long-term observation but such studies are difficult to accomplish. A viable, alternative technique is to use soil chronosequences: soils of different age, situated close together (Huggett 1998). The chronosequence approach has been used by many authors to interpret soil changes

along time scales in many habitat types such as volcanoes (Peña-Ramírez et al 2009), moraines (Sollins et al. 1983), floodplains (Wigginton 2000) and dunes (Salisbury1925, Wilson 1960, Olff et al. 1993, Jones et al. 2008). Most studies use a *synchronic* approach, where sampling is done at the same time but in different adjacent plots. However, this method must be used with extra precaution as it is vulnerable to the confounding effects of high spatial variability (Walker et al. 2010). Using a *diachronic* chronosequence, in which sampling is done repeatedly within the same plot, avoids these potentially confounding effects of high spatial heterogeneity. This method was used, for example, by Abreu et al. (2009) to evaluate the restoration dynamics in the alpine belt of the northern Andes.

In order to study SOM accumulation during primary succession, we used wet dune slack systems on the Dutch Wadden Sea islands because they are relatively young and their history is well known, which allows reconstruction of soil chronosequences (Berendse et al. 1998, Lammerts et al. 1999). In Dutch coastal areas, restoration projects have been carried out for more than 30 years. These projects were aimed at restoring pioneer stages with many rare and endangered plant species. Restoration measures usually included turf stripping and several of these projects have been carefully monitored (Grootjans et al. 2002). Such monitoring programs enable us to assess the exact starting date of vegetation succession after restoration measures, such as turf stripping as well as the natural rejuvenation of dune slacks after sand blow outs. In this study, we present results from chronosequences covering time periods of up to 150 years and data on soil development collected from the monitoring of restoration projects.

Jones et al. (2008) found that differences in SOM accumulation in dune habitats in Wales were primarily associated with climatic factors and the amount of N deposition, and less so with local habitat conditions. However, they did not present information on the hydrological regimes of these wet dune slacks. In our study, we will investigate whether SOM accumulation rates are regulated over time by local factors, such as differences in hydrological regimes, pH, and productivity (measured as above-ground biomass) of the vegetation.

As was discussed above, prolonged flooding can lead to high accumulation rates in wetlands, due to restricted mineralisation, but also to very limited accumulation rates when low productive pioneer species keep the productivity of the vegetation low. We hypothesize that the relationship between flooding duration and accumulation rates in slacks is influenced by the productivity of the vegetation. We, therefore address the following questions:

1) Do slacks with similar flooding regimes show the same accumulation rates?
2) Do other factors, such as soil pH and plant productivity, also affect SOM accumulation rates?

Materials and methods

Site description

This study was conducted in wet dune slacks on four Dutch Wadden Sea islands. Sample sites were selected on the islands of Texel (53° 4' 58.7994"N 4° 49' 58.8"E), Vlieland (53°16’28’’N 4°58’47’’E), Terschelling (53°24’0’’N 5°19’0’’E) and Schiermonnikoog (53°29’29’’N 6°13’43’’E) (Fig. 1).

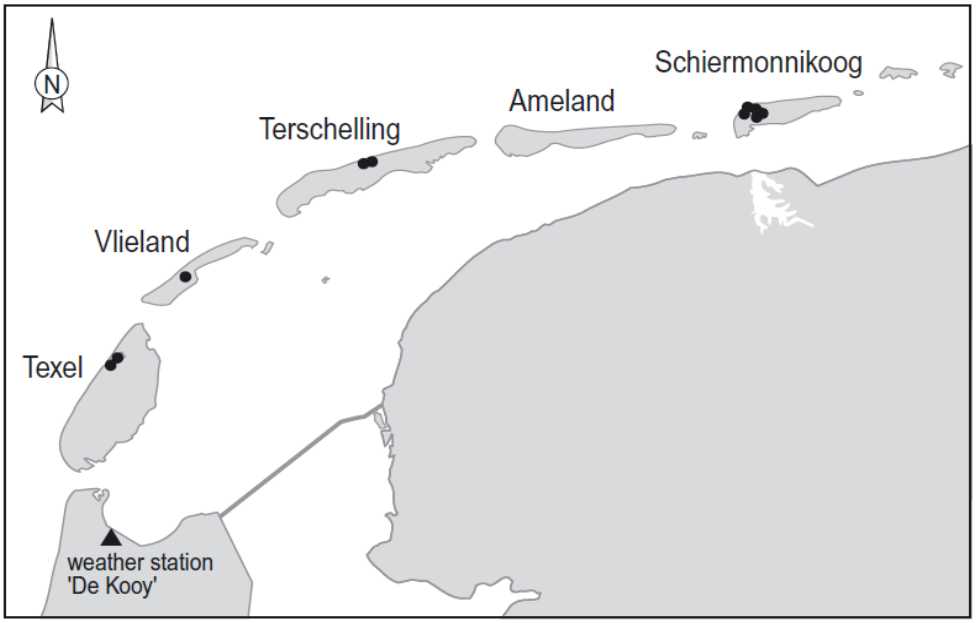

Figure 1. Study sites on the Dutch Wadden Sea Islands

Soil chronosequences

Both types of soil chronosequences addressed earlier were used in this study. We sampled two synchronic chronosequences (Schiermonnikoog and Terschelling) to study SOM accumulation trends in wet dune slacks over longer time scales. The soil chronosequence on Schiermonnikoog consisted of seven sites covering different stages of vegetation succession (16, 18, 20, 52, 60 and 150 years old). The chronosequence on Terschelling included six sites within a secondary dune slack of *ca.* 50 ha. The ages of these sites were 15, 20, 24, 54 and 99 years.

In order to study variation in SOM accumulation rates within soils of the same successional stage, we used several diachronic chronosequences. For this purpose, we sampled four 50-60 year-old sites on Schiermonnikoog: Kapenglop-1, Kapenglop-4, Vuurtorenvallei-1 and Strandvlakte-2. Kapenglop-1 and Kapenglop-4 are located in the same slack and have not been managed since *ca.* 1964. The Kapenglop sites were sampled 4 times with intervals ranging from 5-10 years. In 2010, Kapenglop-1 was still in a pioneer stage, while Kapenglop-4 was in a late successional stage with scrubs and trees up to 5 meters high. Vuurtorenvallei-1 is a 52 year-old blow-out dune slack that has been mown yearly since 2000. This site has been sampled 5 times with intervals of c. 5 years. The vegetation consists of typical dune slack species, as *Epipactis palustris* and *Schoenus nigricans*, with occasional shrubs like *Salix repens* and *Hippophae rhamnoides*. The Strandvlakte-2 is a 50 year-old beach plain that is periodically flooded by sea water and is dominated by *Schoenus nigricans*. It has been sampled 3 times with intervals of 15-30 years.

In addition to these chronosequence sites, we also included data on SOM accumulation from older dune slacks. These ~90 year-old slacks, Buiten Muy-1 (Texel) and Kroon's polder-1 (Vlieland), have been unmanaged from the moment they were first vegetated. The dune slack Buiten Muy-1 originated from a beach plain that was separated from the sea by dune ridge formation between 1920 and 1925 (Adema et al. 2002). The site of Kroon's polder-1 was artificially closed off from the sea in 1924. De Vries (1961) mentions that the dune slack of Kroon's polder-1 had

almost no vegetation in 1941. We included this site because the development of the ecosystem was very peculiar. The site had been dominated by a dense vegetation of *Phragmites australis* during most of the last century. We observed that after about 60 years the above ground biomass of *Phragmites australis* in part of the dune slack declined dramatically due to the increase of a semi-parasitic species *Pedicularis palustris* (De Bakker et al. 2004) Since 2002 several endangered species that are typical for early stages of dune slacks started to increase in numbers as well. This shows that early successional species, such as *Dactylorhiza incarnata, Epipactis palustris* and *Liparis loeselii* can co-exist with late successional species, such as *Phragmites australis*, even on a soil with a thick layer of organic material.

Kroon's polder-1 and Buiten Muy-1 do not represent a real chronosequence because they have not been monitored frequently enough to reconstruct vegetation development. We also added two other sites i.e. Primary valley-3 (Schiermonnikoog; sampled 5 times with intervals of 3-5 years) and Koegelwieck-1 (Terschelling; sampled 5 times with intervals of 3-5 years) that had similar vegetation composition to Buiten Muy-1. Although both these sites were much younger than Buiten Muy-1 (only 20 years old), they had been monitored frequently enough to reconstruct chronosequences.

Estimating soil age

The soil age of sites was determined using various methods. Primarily, we used data from the restoration projects on the Dutch Wadden Sea islands (Grootjans et al. 2002). The age estimation from these projects is very reliable because we know the exact time of turf stripping. The ages for sites without management activities were determined by referring to literature. These descriptions are usually also very precise, since the new formation of dune slacks or enclosure of dune ridges, forming new slacks is well documented on the Dutch Wadden Sea islands. Additionally, aerial photographs and topographical maps were used to assess the starting date of vegetation development at each location.

Sample collection and analysis

Soil samples were taken within one meter of permanent vegetation plots in homogenous vegetation units. At each sampling site, five soil samples were taken from the 0-5 cm layer, and also from deeper layers if the organic layer was thicker than 5 cm, using metal rings with an inner volume of 100 cm^3. The organic matter thickness was measured in the field as the depth of dark-colored organic sand. The soil samples were then homogenized after removing litter, living mosses and roots in the lab. The organic matter was determined by loss of mass after ignition at 550˚ C for at least 15 hours. Soil pH (KCl) was determined by adding 2.5 mL of 1M KCl to 15 g of fresh soil.

Above-ground biomass

Above-ground biomass (peak standing crop) was measured from August to September 2012. All above-ground vegetation was clipped in 5 replicate plots of 0.25 m^2. The samples were oven-dried at 105°C for at least 12 hours. After the drying process, the samples were directly weighed. Forest stand information was obtained from Van der Koppel (unpublished data).

Simulation of water levels

Groundwater level data came from fourteen piezometers with filters at 100-120 cm below the surface. The tubes were placed within 2-20 meters from the soil sampling sites. Groundwater levels were measured every two weeks during 6-10 years. The water level data were collected by the Dutch State Forestry Service (SBB) and

Natuurmonumenten (The Society for the Preservation of nature monuments in the Netherlands). Variations in water levels were simulated using the computer program, MENYANTHES (Von Asmuth et al. 2010). For these simulations, the aforementioned long term water measurements were used. Precipitation data were obtained from weather stations on the respective islands. Groundwater levels were simulated for a period of 25 years (1985-2010). Evaporation data were obtained from the meteorological station, De Kooy. For the present study we used the following (calculated) variables: Mean Higest Level (MHL), Mean Lowest Level (MLL), Mean Fluctuation of levels (MFL) and Inundation Duration (ID). MFL = MLL + MHL.

Data analyses
Differences in SOM contents were tested using the Student-Newman-Keuls test when the sites had three or more sampling years, whereas the Mann-Whitney test was used for the sites with only two years of sampling. We tested for homogeneity of variances using the Levene test and excluded sites with heterogenous variance from further statistical analysis. In order to assess which environmental factors best explained the SOM accumulation in the dune slacks presented in Table 1, we carried out a multiple linear regression analysis. Pearson correlation coefficients were done to test the correlation between variables. Three variables (slack age, above-ground biomass, mean fluctuation) were found not significantly correlated, thus were included in the model. All statistical tests were carried out using SPSS 20.0 (Chicago, IL, USA).

Results

Synchronic chronosequences show similar long-term trends in SOM accumulation
The first chronosequence, established from a series of SOM accumulation (kg/m^2) on Schiermonnikoog (Fig. 2a), showed an increase of 0.26 kg m^{-2} yr^{-1} during the first 60 years of succession, after which SOM accumulation slowed down. The maximal accumulation of 17.5 kg/m^2 occurred at 150 years and this stage was dominated with trees (*Betula pubescens*). Between 60 to 150 years, the SOM accumulation rate had reached a stable state and no further significant increase was observed. The second long-term chronosequence from Terschelling (Fig. 2b) shows a total SOM accumulation *ca*. 2.0 kg/m^2 in the first 20 years. Between 20 to 50 years, there is a steep increase in SOM accumulation, after which SOM levels off. The maximum content of organic matter reached was ca. 10.0 kg/m^2, which is almost half of the maximum amount of SOM at Schiermonnikoog.

The dominating species in this late successional stage were *Salix repens* and *Oxycoccus macrocarpa.* SOM accumulation on the Terschelling chronosequence showed a similar decelerating trend to that measured on Schiermonnikoog, both levelling off after 50-60 years, but the final values of SOM were much lower.

Diachronic chronosequences show very different rates of SOM accumulation between sites
Four diachronic chronosequences aged 50-60 years were sampled in dune slacks on the island of Schiermonnikoog (Fig. 3). Rates of SOM accumulation were remarkably different between dune slacks. The Kapenglop 4, which showed a steep trend during the last 20 years and had the highest SOM content (15.8 kg/m^2) in 2010. Late-successional plant species, such as *Salix cinerea* and *Betula pubescens*, dominated the vegetation of this sampling site. In contrast, Kapenglop 1, which was situated 50 meters away in the same slack, showed a very low organic matter build-up of only

3.5 kg/m^2 after 50 years. The vegetation was still in a pioneer stage (*Samolo-Littoreletum*), dominated by *Littorella uniflora*. Vuurtorenvallei 1 and Strandvlakte 2 both had developed the targeted dune-slack vegetation, with many rare species of the *Junco baltici-Schoenetum nigricantis* community.

These sites showed a slow accumulation rate over the first 30 years (*ca*. 0.1 kg m^{-2} yr^{-1}). However, for Vuurtorenvallei 1, the SOM-accumulation rates appeared to increase gradually until a final amount of 8.0 kg/m^2 after 50 years.
Despite an irregular mowing regime during the last 10 years, the slack was at a mid-successional stage, yet retained many orchid species. On the other hand, Strandvlakte 2 that never been mown, had accumulated a lower SOM with the final accumulation of 3.6 kg/m^2 after 50 years.

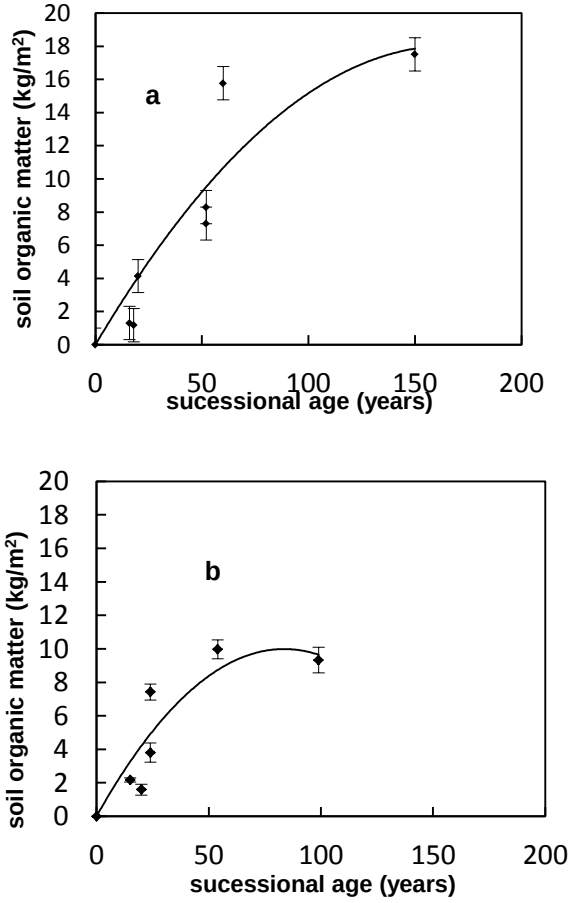

Figure 2. Trends of SOM accumulation from synchronic chronosequence on (a) Schiermonnikoog and (b) Terschelling.

Dominant species and SOM accumulation
In Fig. 4, we present SOM accumulations in slacks dominated by pioneer species *Littorella uniflora*, but with different successional age (Primary Valley 3 and Koegelwick 1 – 20 years old; Buiten Muy 1 – 89 years old). Although Primary Valley 3 and Koegelwick 1 were much younger than Buiten Muy 1, they accumulated similar amounts of SOM (only 1.6 kg/m^2 and 1.2 kg/m^2 of SOM respectively) after 20 years of succession. The accumulation remained low in Buiten Muy 1 (1.9 kg/m^2) even when the slack reached almost 90 years.

In order to see the effect of different dominating species on the SOM accumulation, we compared the four oldest dune slacks that range between 89 to 105 years old (Fig. 5). From the graph we can see that the accumulation rates varied widely between sites with different dominating species. The site with *Littorella uniflora* resulted in an extremely low SOM accumulation, while site with woody species, such as *Betula sp.* showed the highest accumulation.
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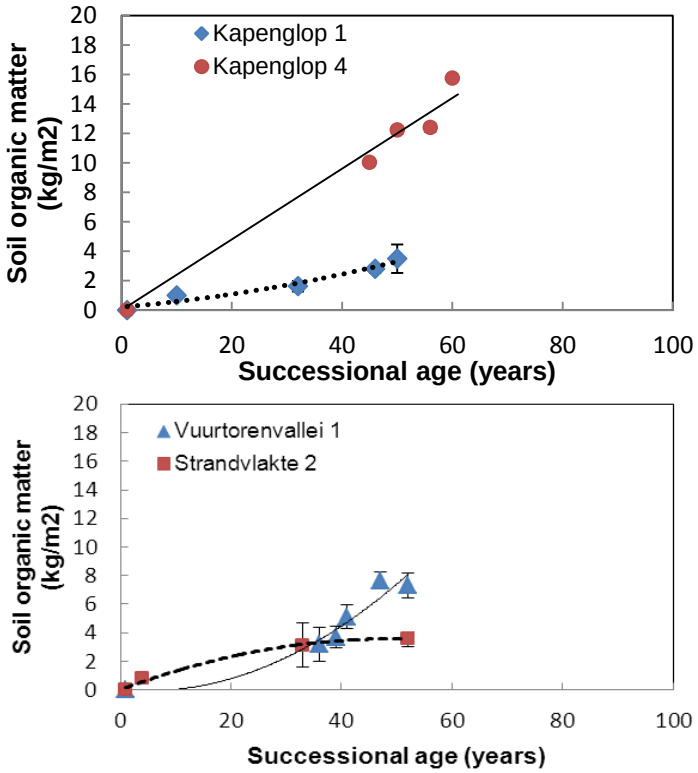

Figure 3. SOM accumulation trend from diachronic chronosequences of various dune slacks on Schiermonnikoog, all aged between 50-60 years. The graph above shows the SOM accumulations in two sites within the same slack (Kapenglop), while graph below shows the accumulations in slack that has been mown (Vuurtorenvallei 1) and without management (Strandvlakte 2).

Local factors (pH, water level regime, and above-ground biomass) in relation to SOM accumulation

In general, pH tended to decline over time for both the synchronic chronosequences (Fig. 6). The soil pH of the pioneer stages (between 15-20 years) was 6.3 and decreased gradually in the late successional stages to a value of 4.2 (150 years on Schiermonnikoog) and 5.1 (90 years on Terschelling) respectively.

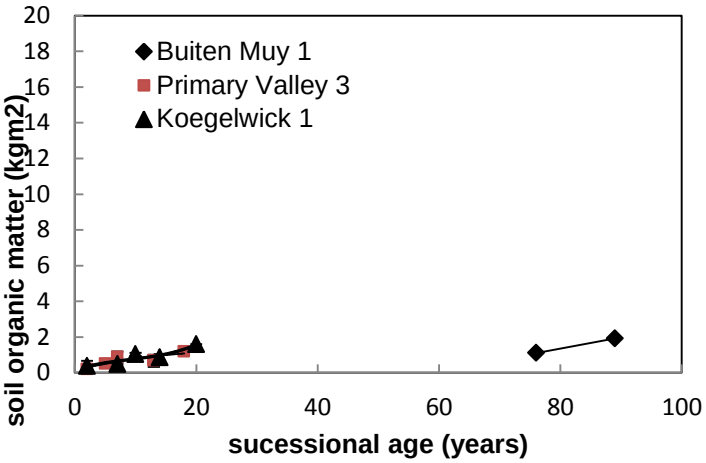

Figure 4. SOM accumulation in slacks dominated by *Littorella uniflora* (Buiten Muy 1, Primary Valley 3, Koegelwieck 1).

Table 1 shows the summary of each variable for the diachronic chronosequences, also including the non-chronosequence sites of Kroon's polder 1 and Buiten Muy 1. In all sites SOM increases with age, except for Buiten Muy 1. As described earlier, this site has hardly accumulated any organic matter for almost a century. In contrast the Kroon's polder-1 site shows a very high SOM while the pH is relatively high. A high SOM accumulation was strongly associated with low water levels in summer (MLG). However, sites with long inundation periods (more than 200 days) showed either very low (Buiten Muy 1) or very high SOM accumulation (Kroon's polder 1), depending on the dominant species at the sites (*Littorella* and *Phragmites*, respectively).

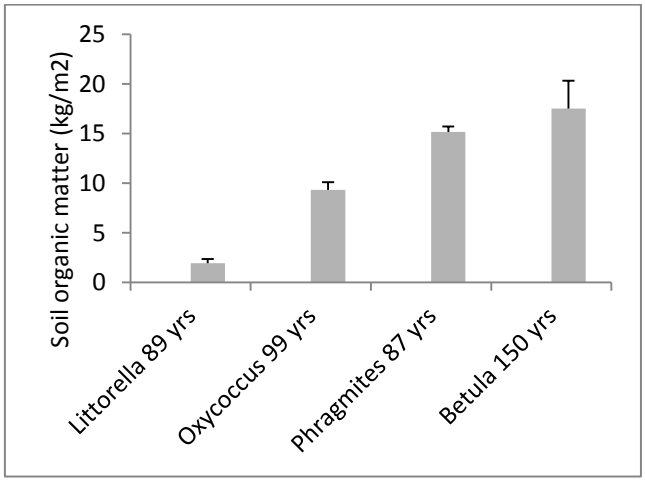

Figure 5. Maximum SOM accumulation among the oldest (unmanaged) sites. The sites have been labelled with their dominant species and age: Buiten Muy 1 on Texel (89 years) Koegelwieck on Terschelling (99 years), Kroon's polder 1 on Vlieland (87 years) and Kobbeduin on Schiermonnikoog (150 years).

In order to assess which environmental factors best explained the SOM accumulation in the dune slacks presented in Table 1, we carried out a multiple linear regression analysis. The results show that only above-ground biomass of the vegetation was significant; Age and mean fluctuation of the water level were not significantly correlated with an increase in SOM (Table 2).

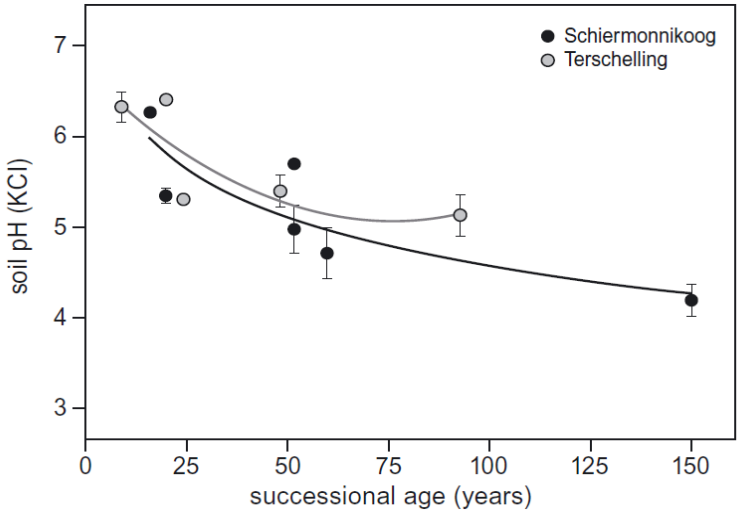

Figure 6. Soil pH (KCl) of Terschelling and Schiermonnikoog chronosequences.

Table 1. Summary of variables for diachronic chronosequence sites: Age – successional age (years); pH(KCl) – soil pH, ID – inundation days i.e. the amount of days per year when the site was inundated; MLG - mean lowest groundwater level below soil surface (m); Biomass- Aboveground biomass, SOM - soil organic matter; and SOM accumulation; MF -Mean Fluctuation i.e.mean lowest groundwater level + mean highest water level (m). The non-chronosequence sites of Kroon's polder 1 and Buiten Muy 1 are also included.

Study site	Age	pH (KCl)	Inundation Duration	MLG	Biomass	SOM	MF
	yrs		days	m	kg/m^2	kg/m^2	m
Primary valley 3	18	6.7	244	0.2	0.42	1.18	0.43
Koegelwieck 1	20	6.4	227	0.28	0.75	1.59	0.48
Kapenglop 1	50	5.8	176	0.48	1.24	3.5	0.62
Strandvlakte 2	52	5.7	192	0.24	0.91	4.65	0.35
Vuurtoren valley 1	52	5	52	0.6	0.64	7.31	0.63
Kapenglop 4	60	4.7	69	0.64	3	15.77	0.66
Kroon's polder 1	87	5.9	186	0.4	2.05	15.15	0.54
Buiten Muy 1	89	6.3	236	0.24	0.2	1.92	0.5

Table 2. Result of multiple linear regression analysis with soil organic matter as dependent variable. Three variables were selected after correlation test; Age, above ground biomass and mean fluctuation. Significant variables are shown in bold.

	β	coefficient	*t*-value	p
Age	0.062	0.272	1.44	0.222
Biomass	**5.076**	**0.801**	**3.74**	**0.02**
Mean fluctuation	1.798	0.033	0.153	0.886

Discussion

The vegetation of dune slacks in coastal areas of north-west Europe normally changes from pioneer stages with sparse vegetation to mature stages dominated by *Betula* forest within 150 years (Olff et al. 1993, Sival and Grootjans 1996). Using a traditional chronosequence approach, in which ecosystems of different age but with a (supposed) similar development history are compared, we found that soil organic matter (SOM) increased more or less linearly during the first 50-60 years and then levelled off to a maximum between 10 and 18 kg/m^2. We also studied ecosystem development in dune slacks using a diachronic chronosequence approach, in which we monitored soil and vegetation development in permanent plots over a period of 20-46 years. We found a large variation in accumulation rates, which varied between 0.02 and 0.26 kg/m^2/year. In general, we found that slacks with a long inundation period accumulated less organic matter than drier slacks, which appears to support our original hypothesis that high water levels, combined with a high pH would lead to slow accumulation of SOM (see also Adema et al. 2002). Some very wet slacks with a very strong discharge of calcareous groundwater showed either very low or very high accumulation rates over a period of *ca*. 90 years. A multiple linear regression showed that variation in SOM accumulation rates were best explained by above-ground biomass of the vegetation. However, this result cannot be generalized. With a greater number of sites the relationships between SOM and hydrological variables would also become significant. But our results show that the accumulation of organic matter does not follow simple rules, but depending on the hydrology and the productivity of the vegetation can result in very different outcomes.

Slacks that were dominated by highly productive species, such as *Betula spp.* and *Phragmites australis,* accumulated a large amount of organic matter over time. This is in line with the results of Smith et al. (2008), who studied SOM accumulation of dune slacks in Massachusetts, USA. These authors suggested that growth of woody plants was the main driver in SOM accumulation in their wetlands. Additionally, wetlands dominated by *Phragmites australis* are among the most productive ecosystems (Westlake 1963, Bakker et al. 1997, Windham and Lathrop 1999). Differences in above-ground biomass of the vegetation may also explain the difference in SOM accumulation in our two synchronic chronosequences; with higher SOM found on Schiermonnikoog than on Terschelling. Previous studies indicated that the aboveground biomass on Schiermonnikoog (Olff et al. 1993) was much higher (3 kg/m^2) than on Terschelling (~1 kg/m^2) (Berendse et al. 1998).

Consequently, factors that increase plant productivity such as nitrogen deposition will also influence SOM accumulation rates as was previously suggested by Jones et al. (2008), when studying wet and dry dune ecosystems in Wales (UK). Since pioneer stages of dune slacks are N-limited (Lammerts and Grootjans 1997), they are very susceptible to atmospheric nitrogen deposition (Lammerts et al. 1998). In the past decades, atmospheric nitrogen deposition has increased significantly in Dutch coastal area (Stuyfzand 1993) and is now becoming a major threat to nutrient-poor habitats (Bobbink et al. 2010, Sparrius 2011). Apart from man-made influences, invasion of certain plant species, such the N-fixing shrub *Hippophae rhamnoides,* may accelerate OM accumulation rates.

Above-ground biomass of the vegetation had a stronger effect than age on SOM accumulation in our study. We found that four older dune slacks of roughly the same age (*ca*. 90-150 years old) had very different SOM accumulation values (Fig. 5). The

very low accumulation rates in the three dune slacks dominated by *Littorella uniflora* (Fig. 4) are quite uncommon and, to our knowledge, have never be found in dune slacks. All sites are very wet; they are flooded for more than 200 days per year. Long inundation periods apparently allow the small pioneer plant species, *Littorella uniflora*, to dominate the vegetation. This species has the ability to decompose its own litter efficiently (Armstrong 1982), hence reducing SOM accumulation. Additionally, this species has ROL capability, and is thus able to reduce nitrogen and phosphate available for competing species (Adema et al. 2002). When inundation periods would decrease, by artificial or natural causes - changes in coastline have immediate consequences for local hydrological conditions (Stuyfzand 1993) – the dune slack may become drier and would enable the invasion of shrubs, like *Salix repens* or tall marsh species, like *Phragmites australis*. The higher litter input of these species to the soil would soon accelerate SOM accumulation and accelerate succession (Walker & Del Moral 2003).

Most of our dune slacks, however, showed similar SOM accumulation trends to those reported by Jones et al. (2008) for dune slacks of the Newborough Warren in Wales. However, some dune slacks on the Dutch Wadden Sea islands showed much higher SOM accumulation rates than the slacks in Wales (Fig. 7).

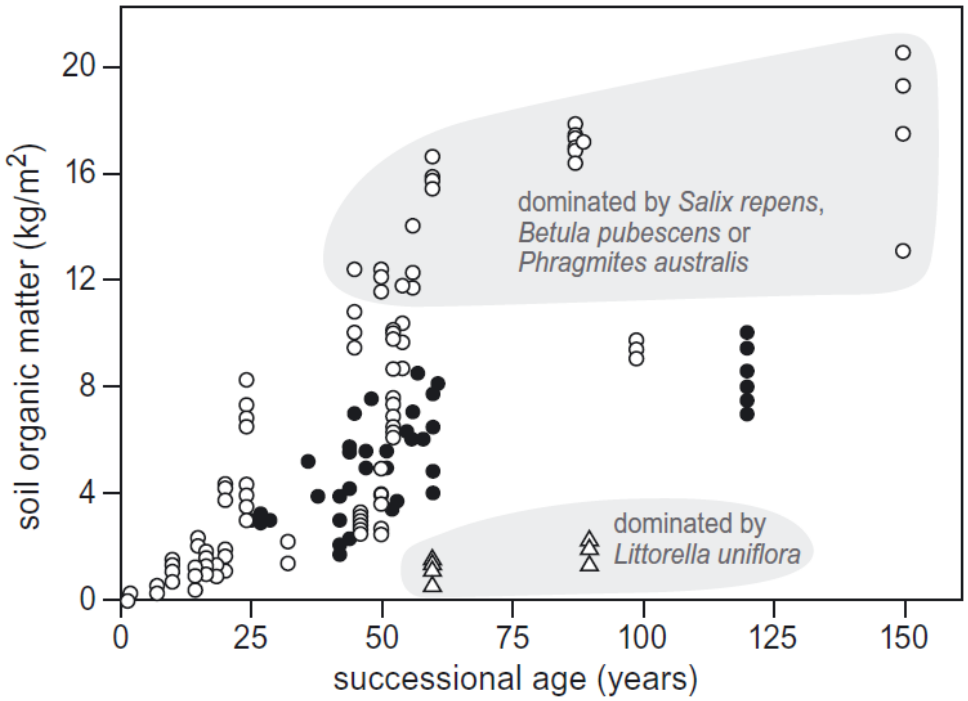

Figure 7. SOM accumulation data from dune slacks in the Netherlands (open symbols) and also the dunes in Wales (black filled circles). Older dune slack with high values of organic matter accumulation are dominated by shrubs or trees (*Salix repens* or *Betula pubescence*), while older dune slack with low accumulation values are dominated by *Littorella uniflora.*

Jones et al. (2008) suggested that turf stripping activities or management measures in the Dutch dunes might explain the observed differences. However, several sites in our study have never been sod-cut or mown but the accumulation rates were still higher than values found in Wales. The differences could be due to differences in management; dune slack in Wales were all mown or grazed. Several of our slacks

were not managed for at least 50 years. The higher accumulation rates in the Dutch dune slack may also reflect differences in annual nitrogen deposition, which are lower in Wales (*ca*.10-15 kg N $ha^{-1}yr^{-1}$ in Wales; Jones et al. 2013, compared to 20-25 kg N $ha^{-1}yr^{-1}$ in the Dutch Wadden Sea islands; Sival and Strijkstra-Kalk 1999). Furthermore differences in hydrological systems and associated differences in water level regimes could be responsible for the observed differences in SOM accumulations rates.

Dune systems in the Dutch Wadden islands are generally much larger than the Newborough dunes, thus generating also more stable water table levels in the dunes slacks, creating wetter conditions. Furthermore, the sandy soils in several dune systems in western England and Wales are more alkaline due to high initial carbonate content compared to most of the coastal dunes included in the present study. Both these factors could lead to higher mineralization rates of organic matter, and lower SOM accumulation rates, in Wales.

An unexpected result was that above-ground biomass drastically declined in a site with very high water levels and prolonged inundation (and very high associated SOM accumulation rates) after 87 years, enabling (re-)establishment of plant species that are typical for early successional stages (*Dactylorhiza incarnata, Epipactis palustris* and *Liparis loeselii)*. This was observed only in one dune slack (Kroon's polder 1), which is influenced by a very regular discharge of calcareous groundwater and where the above ground biomass of *Phragmites australis* was drastically reduced by the spread of the semi-parasitic species *Pedicularis palustris* (see description of chronosequence sites) A possible explanation for the occurrence of a peat layer of c. 20 cm might be the very anaerobic conditions in the topsoil combined with low decomposition rates in *Phragmites* stands due to its highly lignified tissues (Farmer and Morrison 1964, Windham and Lathrop 1999; Meyerson et al. 2000). This results in low nutrient availability. Such conditions favour the spread of *Pedicularis palustris*, a hemi-parasitic species capable of reducing the growth of *Phragmites australis* (Ter Borg 1979, Decleer et al. 2013). From the perspective of nature conservation, the re-establishment of pioneer species in a late-successional stage is quite interesting, since it shows that dune slacks have much in common with natural mires (living peatlands), such as fens that are characterised by high peat accumulation under nutrient-poor conditions. Many rare species, for instance *L. loeselii, S. nigricans* and *P. palustris*, also can be found in nutrient-poor fens, indicating that Kroon's polder 1 is probably developing into a fen.

In conclusion, our results indicate that there is a significant relation between above-ground biomass of the vegetation and the accumulation of organic matter in dune slack ecosystems. This factor however, is closely related to the water level as already discussed above. Even small changes in the hydrology of the system can affect water levels, resulting in drier and more acidic conditions. This leads to the disappearance of basiphilous species and eventually to the encroachment of shrubs. Therefore, it is important to study hydrology of systems at a landscape level in order to understand the successional process in dune slacks and to develop better management strategies. Furthermore, our results support the findings of Adema et al. (2005), who suggested the occurrence of alternative stable states in wet dune slacks. We also found both very high and very low SOM accumulation rates under similar environmental conditions. Whether sudden shifts between such states really occur can only be confirmed when long-term monitoring activities are in place after carrying out restoration projects.

Chapter 3

Alternative stable states in dune slacks revisited: stability of pioneer stages

Bikila W. Dullo, Rohani Shahrudin, Han van Dobben, Eddy van der Maarel, and *A. P. Grootjans*

Abstract

The theory of alternative stable states (ASS) predicts that contrasting ecosystem states can co-exist under the same environmental conditions. In dune slacks, several feed-back mechanisms can lead to the development of ASS. For instance, species capable of radial oxygen loss can modify their micro-environment to their own advantage for a considerable period of time leading to positive feedbacks. In the present study, we analysed the possible occurrence of alternative stable states in dune sacks using long-term data sets of vegetation, soil, water levels and weather conditions. To identify the stability of system states, we combined multivariate analysis techniques, hydrological modelling and regression models. Our results showed absence of long-term stability of pioneer stages; all successional stages converged towards a homogeneous community within 10-12 years, although a large difference in the rate of change was observed depending on hydrological conditions and vegetation types. It is concluded that plant communities in dune slacks can rapidly change in response to relatively small perturbations in hydrological and meteorological conditions. A dry period occurring between wet periods can drastically shift a wet basophile community into a more productive stage, after which the initial community cannot be recovered without human intervention.

Key Words: *adaptive management, hydrology, soil organic matter, restoration, succession*

Introduction

The theory of alternative stable states (ASS) predicts that ecological systems may potentially co-exist in contrasting states under the same environmental conditions (Beisner et al. 2003, Suding et al. 2004). While the debate continues on whether ASS are actually stable or transient (Fukami and Nakajima 2011), many field observations suggest the prevalence of ASS in natural as well as human influenced ecosystems. A mechanism that potentially could sustain ASS is the phosphorus cycle in temperate lakes. Low *versus* high phosphorus input, which is associated with clear water *versus* turbid water states, could cause a regime shift under certain conditions (Scheffer et al. 1997, Carpenter 2003, Folke et al. 2004). The occurrence of ASS in tropical lakes has also been suggested by observations, experiments and models, where a stage with free-floating water plants and a stage dominated by submerged plants occur as alternative states (Scheffer and Carpenter 2003). Similarly, ASS have been suggested to occur in wetlands, estuaries and coastal seas (Kautsky et al. 1986, Elmgren 2001, Gunderson 2001, Van der Heide et al. 2013); in savannahs (Kelly and Walker 1976, Anderies et al. 2002); coral reefs (Nystrom et al. 2000, Jackson 2001, Jackson and Johnson 2001, Nystrom and Folke 2001); and other ecosystems (Folke et al. 2003 , Folke et al. 2004). However, only a few experimentally induced ASS have been reported (Van de Koppel et al. 2002, Petraitis and Dudgeon 2004, Schroeder et al. 2008).

Bossuyt et al. (2005), working in Belgian and French dune slacks, suggested that intraspecific processes can lead to non-random vegetation composition. Even though the authors did not specifically mention alternative stable states in their study, they indicated that species may modify their environment and create a positive feed-back mechanism, thus forming an aggregated community. Adema et al. (2002), working in dune slacks on the Dutch Wadden Sea islands, showed a clear case of the existence of ASS in dune slacks, where a pioneer stage co-existed with later successional stages over a period of more than 80 years. They suggested three possible feed-back mechanisms that could lead to ASS (Adema et al. 2002; see Fig. 1). The first mechanism is associated with enhanced nitrogen loss, caused by radial oxygen loss (ROL). Wetland species with ROL capabilities, such as *Littorella uniflora* are able to release large quantities of oxygen into a predominantly anoxic environment *via* their root system, thus stimulating nitrification in the root zone. In a system with groundwater flow, the nitrate produced can be transformed into N_2 by micro-organisms when the water leaves the root zone. This causes loss of nitrogen because N_2 is a gas and can escape to the atmosphere (N-loss through coupled nitrification-denitrification; Reddy et al. 1989, Engelaar et al. 1991, Bodegom et al. 2005). The second mechanism discussed is sulfide toxicity caused by microbial mats sealing off the surface layer of the slack and facilitating the development of high sulfide concentrations. In this case, sulfide prevents the establishment of productive species because in dune slacks, most of these species cannot cope with high levels of sulfide; thus pioneer species, which are often adapted to this environment, are favored (Van Gemerden 1993, Lamers et al. 1998). The third possible mechanism involves productive species growing in nutrient-rich conditions (Olff et al. 1993, Lammerts et al. 1997, Walker and Moral 2003). Such species store a large amount of nutrients in the soil organic matter (SOM) or in their biomass, which can be retrieved again during the next growing season. This mechanism favors late successional species, such as *Salix* and *Betula* species (Ernst et al. 1996). This development towards dune woodland can be considered an alternative stable state (Beisner et al. 2003).

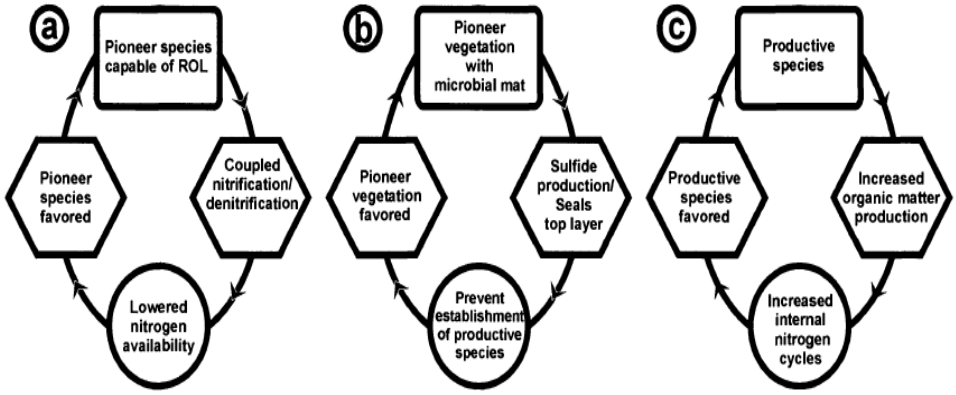

Figure 1. Three possible feedback mechanisms are recognized in calcareous wet dune slacks: **(a)** Enhanced nitrogen loss; **(b)** Sulphide toxicity; and **(c)** Nutrient accumulation in an internal cycle. The diagram was adopted from Adema et al. (2002).

In this study, we focus on evidence for alternative stable states (ASS) in dune slacks. We attempt to unravel the long-term trends and shifts in plant communities by analyzing a long-term data set collected from permanent quadrats. Our objective was to detect spatio-temporal changes in the vegetation and soil parameters that point to community shifts toward one system state (transience) instead of several or at least two other system states (stability). In this paper, long-term monitoring data (from 1991 to 2008) in combination with a chronosequence of successional stages (up to *ca.* 90 years) were used to investigate whether alternative stable states in dune slacks are stable or in transition. Vegetation changes were analyzed using a similarity index in species composition as a measuring tool for ASS.

We assume that if a local plant community has a permanently low similarity with neighboring community types under comparable abiotic conditions that community may be in an ASS. An increasing similarity over time with other communities under comparable abiotic conditions can be considered as evidence for the absence of ASS. Accumulation rates of organic matter in dune slacks were used as another indicator for the occurrence of ASS; low accumulation rates over a rather long period of time (*c.* 30 years) point to restricted succession and the presence of enhanced nitrogen loss as depicted in Figure 1a (Adema et al. 2002, 2005).

From an applied perspective, the occurrence of stable states in pioneer stages of dune slacks is relevant for nature conservation. If natural (hydrological or meteorological) conditions can prolong the lifespan of these pioneer species, it would save money that is now used in regular management (mowing and sod cutting, Grootjans et al. 1998, Bakker et al. 2006, Bakker et al. 2007, Chapter 2).

Methods

Study area

The study site is located on the Wadden Sea Island of Terschelling, the Netherlands, in a large dune slack known as Koegelwieck (53º24'N, 5º20'E). A detailed description of the study site is provided in chapter 3 of this thesis. The Koegelwieck is a secondary dune slack of about 50 ha, which was formed between 1825 and 1865

(Van Dieren 1934) as a result of intensive sand blowing. In this dune slack, five sites have been monitored with known ages, vegetation, soil and hydrological parameters. The vegetation records from each of these sites form a chronosequence (see Shahrudin et al. 2014, chapter 3). Top soil removal was carried out in the Koegelwieck dune slack several times experimentally in order to see if target species could re-establish populations after they had been lost due to rapid succession.

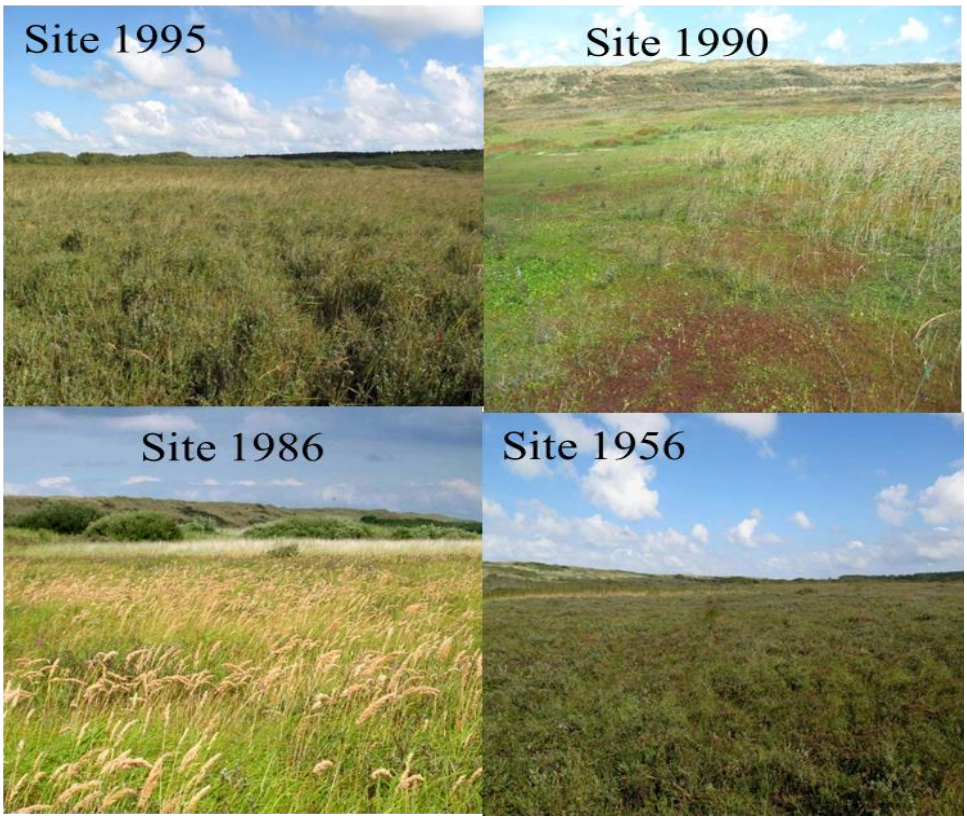

Figure 2. Four chronosequence sites investigated since 1956, 1986, 1990 and 1995. Photographs of sites 1986 and 1990 were taken in 2004; those of sites 1956 and 1995 were taken in 2009 (all photographs taken by A. P. Grootjans).

The vegetation of the oldest site is almost 90 years old; vegetation development started after sod-cutting in 1920. At the next oldest site, vegetation dates from 1956, when this site was sod-cut. More recent sod-cuttings were carried out in 1986, 1990 and 1995, respectively, in order to restart vegetation succession (Fig. 2).

Vegetation, soil organic matter and pH data

Detailed descriptions of the sampled sites and continued monitoring activities in our study area since 1990 have been well documented elsewhere (Lammerts et al. 1997, Grootjans et al. 2002, Sýkora et al. 2004). Soil samples were collected in 1991, 1993, 1996, 1999, 2003 and 2008. They were dried and the percentage of organic matter was determined by loss through ignition. Similarly, the soil pH (KCl) was measured in the laboratory using 17.5 g of fresh soil dissolved in demineralized water and mixed thoroughly for two hours. The peak standing crop of each chronosequence site was measured in September 2009 by cutting the total above-ground vegetation (20 x 20 cm; 7 replicates) in sampling plots located next to the permanent plots. All vegetation samples were dried in an oven for 12 hours and the dry weight was measured.

Statistical analyses were carried out using IBM SPPS statistics 20 (repeated measure analysis).

Each year from 1991 to 2008, 26 plots were surveyed and all plant species were recorded using the cover-abundance scale of Londo (1975). Changes in species composition in the four chronosequence sites were analyzed using CANOCO 4.5 software (Ter Braak and Šmilauer 2002). The underlying latent gradients in the vegetation data and the temporal changes in each permanent plot during subsequent years were analyzed using Detrended Correspondence Analysis (DCA). For DCA, the cover abundance scale values (measured as percentages) were square-root transformed in order to reduce the effect of dominant species on the analysis. The method used to detrend axes was detrending by segments, and rare species (n < 3) were down-weighted. Based on the length of gradients and eigenvalues of axes, we were able to interpret some axes as latent variables, based on ecological characteristics of the species. In the DCA diagram, average positions of sampling points of each site for the corresponding years were calculated and used to visualize changes in vegetation. The effects of soil pH and soil organic matter were derived from related work in Chapter 3 of this thesis. Here, they are presented graphically to demonstrate the changes that took place over the years.

The similarities within each site (changes in plant species composition over time at the same location) and among different sites over the years (changes over time at different places) were calculated with the Diserud–Ødegaard similarity index (Diserud and Ødegaard 2007). This index is a modification of the similarity index of Sørensen (1948) and is very suitable for comparing species composition data set of different sites; it can calculate similarities within and among two or more communities.

To give an example of comparing three relevés, A, B and C, the index of similarity is given by the following equation:

$$\frac{ab + ac + bc - abc}{a + b + c} \qquad \textit{Equation 1.}$$

where '*a'*, '*b'* and '*c'* are the numbers of species found in the relevés A, B, & C, respectively; and '*ab*' is the number of species common to A and B; '*ac'* is the number of species common to A and C; '*bc'* is the number of species common to B and C; and '*abc'* be the number of species common to all 3 relevés.

When calculated using the above formula (Equation 1), this index will be exactly 2/3 if all three relevés share exactly the same species and 0 when all three relevés have no species in common. Diserud and Ødegaard (2007) suggested that the index value can best be multiplied by 3/2 in order to obtain a range from 0 and 1 and, according to the authors, this equation can be expanded to compare more than three relevés at the same time.

In this paper, we used the Diserud and Ødegaard similarity index to make two types of comparisons. We first made comparisons in species composition within the same permanent plot over time. To detect changes, we calculated the similarity index between vegetation survey data taken at the start of monitoring (1956, 1986, 1995 and 1990, respectively, for the four chronosequence series) and at four different sampling times. This would enable us to see if the permanent plot remained stable or showed changes in species composition over the years. We then compared changes between two chronosequence sites: 1956 with 1986, 1956 with 1990, 1956 with

1995, and 1986 with 1990. For this purpose, we calculated similarity indices between two permanent plots from each chronosequence sites. Since the two compared plots from the chronosequence sites are permanent plots, we repeated similar calculations for the period the vegetation had been recorded. These comparisons of different chronosequence sites with one another will help us discover whether the species composition of these sites converged or diverged over time.

After the indices of similarity were computed, these indices were plotted against the time interval over which they were calculated. In order to test whether there was a shift in community composition as a result of changes in species composition, we tested if a significant change over time existed in species composition. Our null hypothesis was: there is no change in species composition and thus no shift in community types, which means similarity indices should not indicate any significant differences. Thus, the test of significance was based on the assumption that the similarity index should not change significantly over the time interval for which indices were calculated if the communities had not changed. To test this, we used a t-test in SPSS 20.

Results

Vegetation dynamics

The relative positions of 29 species are presented in a DCA ordination diagram (Figure 3). The first DCA axis explains 25% of the species variance; axes 1 and 2 combined explain 34.7%, while all axes together (1–4) explain 42.2% of the variation.

The gradient length of the first axis is 3.48 and for the remaining axes the length is < 2.0. Eigenvalues are 0.55, 0.21, 0.12 and 0.05 for axes 1–4, respectively. The positions of the species in the ordination space are related to variation in wetness and time, which are the latent variables underlying the first and the second axes, respectively (Table 1).

Table 1. Summary of results of the Detrended Correspondence Analysis for ordination graph shown in Figures 3 and 4.

Axis	1	2	3	4	Total inertia
Eigenvalues	0.55	0.21	0.12	0.05	2.21
Lengths of gradient	3.48	1.66	1.83	1.46	
Cumulative percentage variance of species d	25	34.7	40	42.2	
Sum of all eigenvalues					2.21

The relative positions of all sites of the chronosequence sequences and monitoring years are given by the DCA diagram (Figure 4). Two shifts can be noticed. To the left, small changes in vegetation composition can be observed in the old sod-cut experiment (1956) over time; plots 'moved' in the direction of the top of the diagram. The 1986 site, found in the middle, shifted left to more acid conditions as early as 1996 and, within 17 years, resembled the 1956 site. To the right, the very wet site that had been sod-cut in 1990 also showed relatively little change until 2004.

Between 2004 and 2008, a relatively large change occurred towards more eutrophic conditions at the top of the diagram (see Fig 3; large changes can be observed in the 1986 and the 1956 sites). The youngest site from 1995 changed towards a more productive stage between 2001 and 2008, also moving up the diagram, with rapid increases of *Salix* and *Phragmites*.

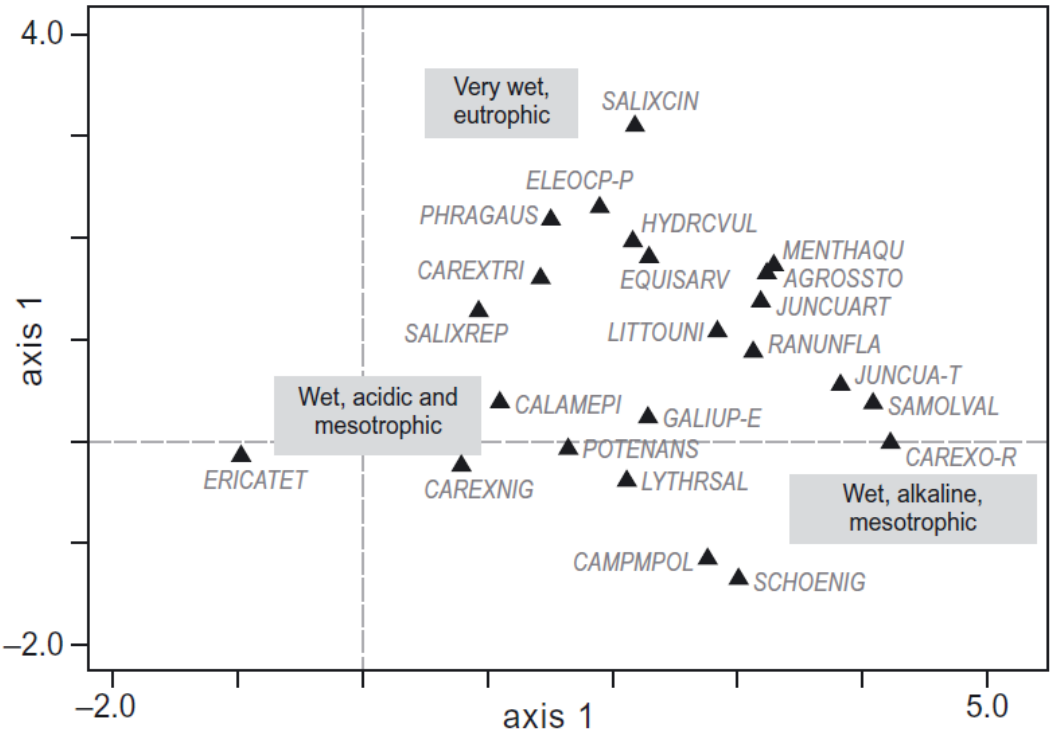

Figure 3. Distribution of plant species in ordination space (DCA). *AGROSSTO = Agrostis stolonifera, CAREXNIG =Carex nigra, CAREXO-R = Carex oederi s. oederi , CAMARPOL = Camarops polysperma, CAREXTRI = Carex trinervis, CALAMEPI = Calamagrostis epigejos, EQUISARV = Equisetum arvense, ERICATET = Erica tetralix, GALIUP-E = Galium palustre, HYDRCVUL = Hydrocotyle vulgaris, JUNCUA-T = Juncus alpinoarticulatus s. atricapillus, JUNCUART = Juncus articulates, LITTOUNI = Littorella uniflora, LYTHRSAL = Lythrum salicaria, MENTHAQU = Mentha aquatica, OXYCOMAC = Oxycoccus macrocarpos, PHRAGAUS = Phragmites australis, POTENANS = Potentilla anserina, RANUNFLA = Ranunculus flammula, SALIXCIN = Salix cinerea, SALIXREP = Salix repens, SAMOLVAL = Samolus valerandi, SCHOENIG = Schoenus nigricans.* Only species with a cover of at least > 1% are shown. These species range from acidophilous heathland species (left bottom corner) to wet pioneer species (right bottom corner), and scrub species including *Salix* of old dune slacks (top).

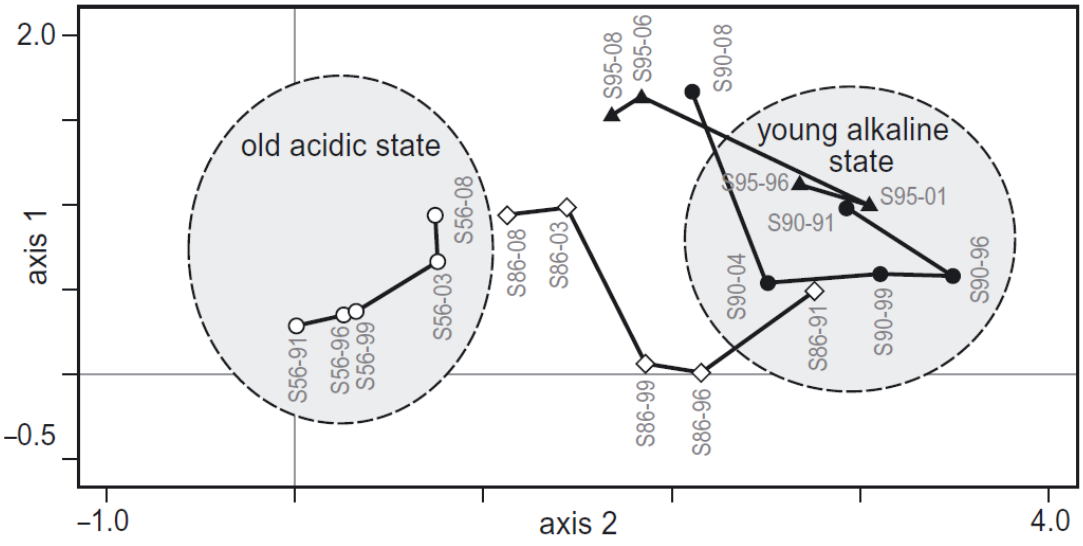

Figure 4. Site distribution graph (DCA**)** corresponding to the species distribution graph given in Figure 3 above. The large circles indicate the boundaries of two states (an older acidic state and a younger alkaline state), which can be considered as alternative stable states.

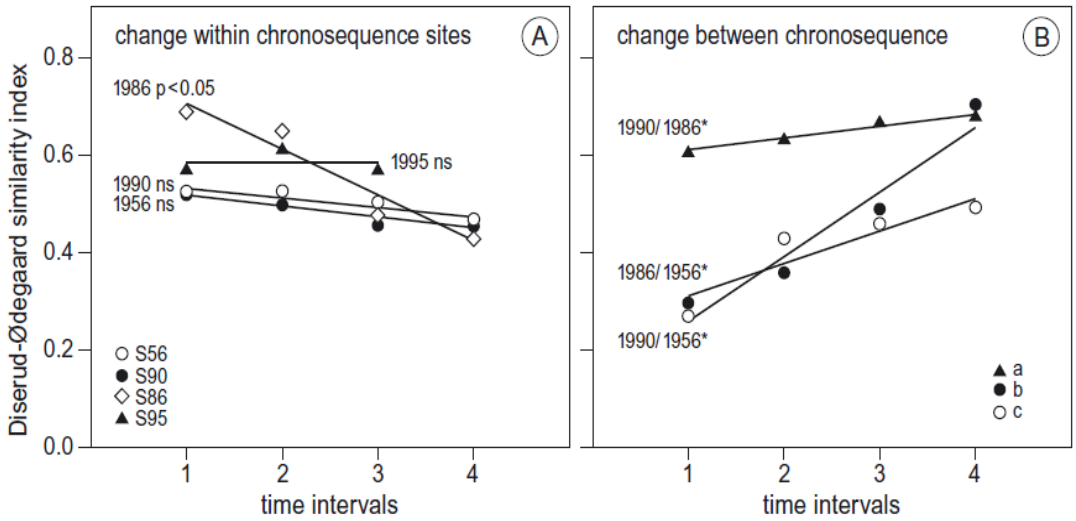

Figure 5. a. Changes in species composition over time (compared to the start of the monitoring) of four chronosequence sites (1956, 1986, 1990 and 1995) using the Diserud–Ødegaard index. Only changes within the site sod-cut in 1986 were significant. **b.** Changes in species composition between chronosequence sites (1956 compared to 1986, 1956 compared to 1990, 1956 compared to 1995, and 1986 compared to 1990) over time. The site that was sod-cut in 1986 came to closely resemble the 1956 site at the end of the monitoring period. The 1990 site (pioneer) did not develop toward either the 1986 site (target community) or the 1956 site.

Temporal changes (from 1991 to 2008) in the similarity index within chronosequence sites are given in Figure 5a. The result is an indication of how a given chronosequence site changed over time in terms of species compostion. The statistical significance of the changes are indicated in the graphs.
The only chronosequence site that showed a significant change in species composition was site 1986, with a similarity index dropping from above 70% in the beginning to about 45% at a later stage.

Two chronosequence sites, 1956 and 1990, showed a slight decrease in species similarity as shown by the slope of the graph (a negative slope indicates a decrease and a positive slope indicates an increase in similarity of species composition), but for both sites, the similarity index varied from just above 50% to about 48%, indicating that this sites maintained at least 50% of all its species over time. The youngest site from 1995 had a high similarity index (about 60%) over time.

The similarity index (Figure 5b) among the chronosequence sites shows a general increasing trend over the sampling period. Comparison between the chronosequence sites of 1986 and 1990 revealed a 60% similarity already when their respective vegetation stands were only five years old. Despite this high early stage similarity, however, their index of similarity only slightly increased to about 65% over a longer period of time. A similar comparison between site 1956 and site 1986 shows a rise in similarity from about 25% in the beginning to about 65% when the vegetation stand at site 1986 was only 17 years old and that of site 1956 was 47 years old. A similar comparison between site 1956 and site 1990 shows less pronounced similarities between the two sites: less than 30% in the beginning and about 40% at a later stage. The younger site of 1995 attained a much higher similarity with site 1956 in only ten years. Their similarity index increased from about 35% at age six of the vegetation to above 80% at age ten.

Changes in environmental factors
Organic matter (OM) accumulation, soil pH and peak standing productivity in five sites in the Koegelwieck are shown in Figure 6. The sites vary in OM accumulation. In the oldest site, sod-cut in 1920, it was *ca.* 7 kg/m^2 in 1991, increased to *ca.* 9.7 kg/m^2 between 1991 and 1999, and decreased slightly after that. The samples (n = 8) showed a high variation, however. The second oldest site, sod-cut in 1956, showed a linear increase between 1991 and 2008 from 6.5 kg/m^2 to ca. 10 kg/m^2. For site 1986, for which we sampled two sub-sites, soil samples taken in 1991, *i.e.* 5 years since top soil removal, showed an OM accumulation of ca. 3.5 kg/m^2, after which the OM values of the sub sites started to deviate from each other. The younger sites, 1990 and 1995, accumulated very little OM compared to the older sites over 18 years and 13 years, respectively.

The corresponding soil pH data in Figure 6B show a general trend of lower pH at older sites and higher pH at younger sites. At the older sites, 1920 and 1956, soil pH data measured in 1991 were below 6. The pH values in the 1986 sub-sites were similar, although organic matter contents were different (Figure 6a). Five years after top soil removal, the pH at this site was still above 6 but it dropped to around 5 over the 18 years that followed. In the younger sites (1990 and 1995), the pH also decreased but remained above 6.

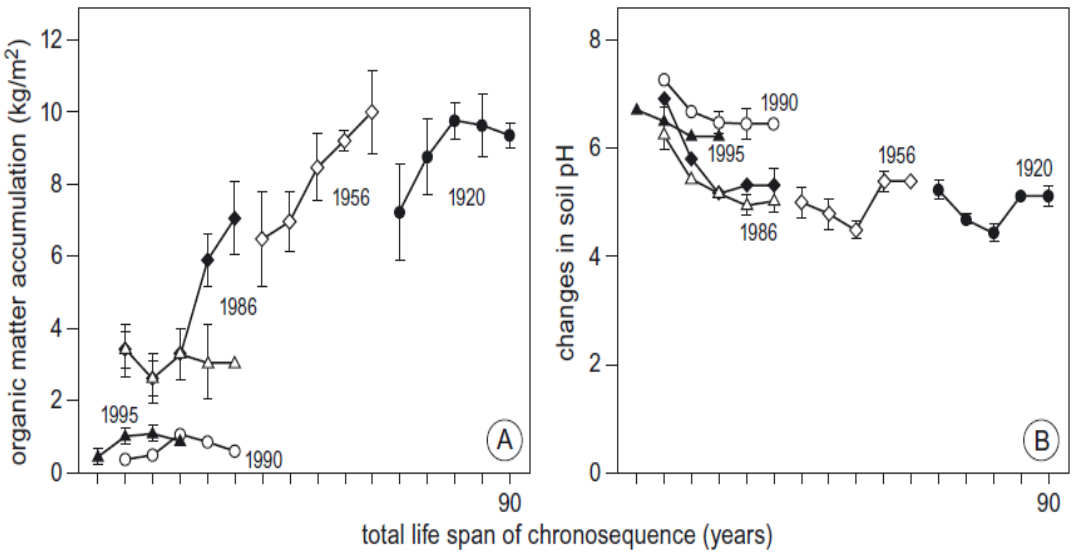

Figure 6. Changes in organic matter content (A) and pH (B) of the top soil over time in 5 chronosequence sites at Koegelwieck. The data points from left to right represent data collected over time from 1991 to 2008. For site 1995, the first sample was taken in 1996. The diagram shows younger sites to the left and older sites to the right. The age displayed on the x-axis refers to known age of vegetation in the dune slack, tracing back to 1920.

The above-ground biomass measured in September 2009 (Figure 7) did not show much variation between the chronosequence sites. Only the 1990 site (at pioneer stage) still had a low peak standing crop, which was significantly lower than that found at other chronosequence sites.

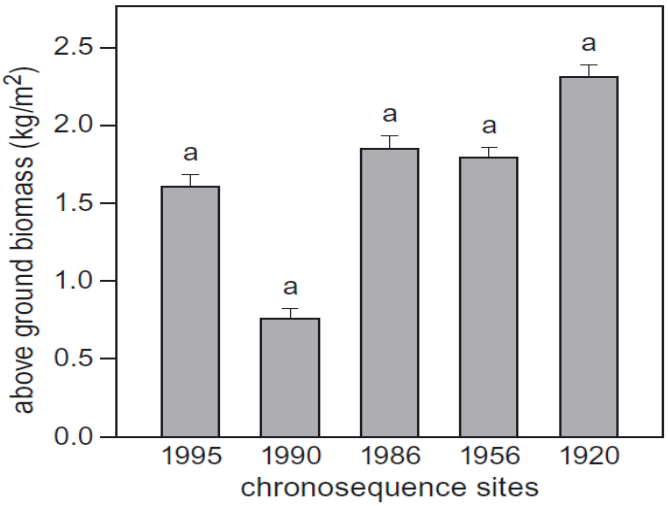

Figure 7. Peak standing crop at the chronosequence sites in the Koegelwieck dune slacks. Significant differences indicated by different letters; only the chronosequence site initiated in 1990 was significantly ($p<0.005$) different from the rest of the sites.

Discussion

A convincing example of the occurrence of alternative stable states (AAS) during succession in dune slacks on the Dutch Wadden Sea island of Texel was provided by Adema et al. (2002). They found that pioneer stages (*Littorella uniflora*) could co-exist with later successional stages (*Salix repens, Phragmites australis*) and proposed several mechanistic explanations regarding how ASS could develop and maintain themselves under natural conditions.

The main question in our study on the long-term development of dune-slack vegetation on the Wadden island of Terschelling was whether alternative stable states (ASS) could also develop after restoration measures, notably sod-cutting and hydrological manipulations, to increase groundwater discharge to a degenerated dune slack, Koegelwieck.

Stability in species composition

In the present study, we found that the stability of a vegetation type that had developed after restoration measures depended on the hydrological and climatological conditions that prevailed immediately after restoration. Our analyses showed that early pioneer stages (sites 1986 and 1990) may first diverge into very different vegetation types. Site 1986, with many rare and protected plant species, was quite unstable and quickly shifted into a late successional stage after only 5 years of succession. The ordination diagrams show that the oldest site (1956) and the young site dominated by *Littorella uniflora* (1990) were relatively stable (Figure 5). In contrast, site 1986 shifted rapidly from a community type rich in basiphilous species towards a community with more productive and acidophilous species, such as *Calamagrostis epigejos, Oxycoccus macrocarpa* and *Phragmites australis*. The similarity within this site, as compared with the starting situation, decreased during succession, because the early colonizers had disappeared from this site. This may have happened because certain colonizers became dominant and outcompeted other early colonizers or because most early successional species had been replaced by later successional species. The second explanation is more likely because the similarity index of site 1986 with the older site from 1956 increased during succession. Site 1990 became more dominated by wet species, such as *Littorella uniflora* and *Mentha aquatica.* This site seemed more stable than site 1986 when considering the within-site similarity values. Site 1990 had a low similarity with the old site from 1956, probably because of the absence of the late-successional species common in site 1956. The vegetation at the younger site from 1995 was similar that at site 1956 during an earlier stage. A sudden shift in vegetation composition probably occurred at this site, maybe due to the occurrence of the dry years following sod-cutting.

Stability of environmental factors

The strong relationship between age of a young successional stage and amount of soil organic matter (SOM) during the first 50 years of succession has already been mentioned by Olff et al. (1993) and Sival (1997). However, this relationship is not always linear, especially during the build-up phase. For example, Jones et al. (2008) showed that for dunes systems in Wales (UK),, the SOM increase followed a sigmoidal pattern over time. In dune slacks, discharge of calcareous groundwater and the presence of calcareous substrates appear to be responsible for the initially slow increase of organic matter during the pioneer phase (Sival and Grootjans 1996, Kooijman et al. 1998, Kooijman and Besse 2002, Kooijman 2008).

SOM accumulation in the Koegelwieck also showed a clear sigmoidal curve. In fact, only one site (site 1986) showed a rapid increase during the monitoring period. However, another sampling site only a few meters away did not show this rapid increase during the monitoring period that was analyzed. This difference could be attributed to the specific characteristics of their locations within the dune slacks. There was about a 20 cm site height difference between these two sub-sites, which would result in a difference of water level between them. Indeed, the sub-site with the lower elevation accumulated less organic matter. From evidence here and also that collected from site 1990, which was the lowest site in the dune slacks, we may state that higher water levels lead to lower organic matter accumulation. This phenomenon has been extensively discussed elsewhere (Bakker 2005, this thesis, chapter 2 and 5).

Stable or transient?

We were able to test assumptions about the occurrence of alternative stable states because the Koegelwieck dune slack provided a unique opportunity to study spatial and temporal aspects of ecosystem development. The combination of regular vegetation monitoring in (permanent) plots with precisely known ages and regular sampling of soil parameters within this chronosequence has provided us with this opportunity. Our research has shown that restoration measures do not necessarily lead to the development of an ecosystem that can be considered stable over a considerable period of time as was shown for a natural dune slack on Texel (Adema et al. 2002). In Koegelwieck, seemingly similar restoration measures led to different vegetation types, with different stability characteristics, depending on the hydrological characteristics of the time during which the measures were carried out (wet/dry periods).

Most pioneer stages were not resilient and could not return to the same state after relatively small disturbances. For instance, site 1995 in our study shifted toward a more productive stage as a consequence of relatively dry conditions shortly after the site was sod-cut (1996). However, this shift in vegetation has not yet been reflected in the measured soil parameters, although it had already been apparent in the above-ground biomass after 13 years. In contrast, the older site sod-cut in 1990 remained at a rather stable pioneer stage, with almost no accumulation of organic matter and low above-ground biomass for more than 14 years. However, after a period of relatively wet years (2000-2008; Chapter 3), the pioneer stage with *Littorella uniflora* was slowly replaced by a more productive stage dominated by *Phragmites australis*.

We found that changes in vegetation preceded changes in soil factors, such as soil OM and soil pH, and that relatively small changes in species composition could trigger big changes when accompanied by unpredictable changes in hydrological or meteorological conditions (see also chapter 3). The competitive advantage that early successional species may have during the first years following restoration (Bakker et al. 2006, 2007) may not always last very long. Even small changes in vegetation composition could lead to drastic shifts in vegetation when exogenous environmental factors suddenly changed (Grootjans et al. 2001). For instance, one dry year in the summer could shift wet basiphilous vegetation into moist vegetation dominated by species such as *Oxycoccus macrocarpa* (Chapter 3 of this thesis).

This would benefit nature conservation, because pioneer stages that could maintain themselves in a stable state without additional management measures would be a cost-efficient way to conserve threatened plant species for an extended period of time.

Acknowledgement
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Chapter 4

Population structure and germination capacity of two pioneer dune slack species

Rohani Shahrudin

Abstract

The aims of this study were to investigate the effects of management and abiotic factors on the population structure of two pioneer species in wet dune slacks on a Dutch Wadden Sea island. The population structures of these species, *Schoenus nigricans* and *Pedicularis palustris,* were described by using three different life-history stages: seedlings, juveniles and adults. Additionally, a germination experiment was carried out for both species to test for differences in the seed viability between different aged populations. Finally, a field experiment was carried out to test if *P. palustris* seeds were able to germinate under inundated conditions. The assessments of the population structures showed that dune-slack age was an important factor for *S. nigricans*; seedlings formed the most abundant class in the populations at pioneer sites. However, the age of the slack did not influence the population structure of *P. palustris* but abiotic factors were important; drier sites contained more seedlings compared to wetter sites. All *S. nigricans* sites were characterized by high pH (>6.0) and low organic matter content (<0.1kg/m^2) whereas *P. palustris* sites showed a large variance in pH and organic matter content (pH: 3.8 - 7.1; organic matter: 0.08 - 1.8 kg/m^2). The germination tests revealed that for *S. nigricans*, seed viability was highest in the oldest population (50 years old), but no differences in seed viability were found between the different aged populations of *P. palustris*. The field experiment revealed that *P. palustris* seeds were not able to germinate under the following conditions: (i) when a site was inundated, (ii) when a site was too dry and, (iii) when seeds were floating.

Introduction

Young wet dune slacks have become scarce nowadays due to many factors, such as decreasing ground-water levels, fixation of dunes and increased nitrogen deposition (Van Dijk & Grootjans 1993). This degradation of habitat quality for typical dune-slack species has consequently affected many species, including several Red List species. Management efforts such as sod removal, mowing and grazing have been among the common practices used to restore degraded slacks. The reasons behind the success or failure of several restoration projects have been discussed at the level of species and vegetation (Grootjans et al., 2002, Van der Hagen et al. 2008). However, studies on the effect of restoration at the population level are still scarce. Therefore, this study was initiated to investigate the effect of management activities on the population structure of two selected pioneer dune slack species, *Schoenus nigricans* L. (Cyperaceae) and *Pedicularis palustris* L. (Scrophulariaceae).

Ernst & van der Ham (1988) showed that populations of *S. nigricans* had more regeneration potential in perturbed areas compared to stabilized areas, whereas Petru & Leps (2000) found that the number of seedlings and rosettes of *P. palustris* increased in plots after removal of the litter layer and bryophytes. These activities increase the availability of safe sites for seeds to germinate. However, abiotic factors that are related to these activities also should be considered. Abiotic factors, such as soil organic matter, pH and water level, may affect the germination and establishment of pioneer species. An increase in organic matter has been shown to lead to a decrease in the seedling survival (Mudrák & Lepš 2010), fruiting (Van Hulst et al. 1987) and density of hemiparasites (Westburry & Dunnett 2007). Additionally, soil pH was found to be among the factors that affect the number of capsules formed in *P. palustris* (a hemiparasite) by Schmidt & Jensen (2000). Finally, seeds of *P. palustris* have been shown not to germinate under inundated conditions by Ter Borg et al. (1980). Such studies have shed some light on which environmental factors are possibly decisive for regulating populations of *P. palustris* in areas with strongly fluctuating water levels.

The main aim of this study was to analyze the population structure of *S. nigricans* and *P. palustris* in order to gain insight into the long-term effects of management on populations of these species. We selected these two species because they normally co-exist in the same slack. To address possible causes underlying population structure, such as recruitment potential, germination experiments were done to test the viability of seeds from each population. Additionally, a seed-sowing experiment also was also done for *P. palustris* to test germination ability under different inundation levels in the field. The questions we seek to answer are: (i) Do management activities affect the population structure and also population viability? (ii) How do populations differ in the soil pH and organic matter of their environment? (iii) Does prolonged flooding prevent germination in *P. palustris* populations?

Materials and Methods

Study species

Schoenus nigricans is usually found on acidic and alkaline peat soils, mineral soils rich in organic matter and calcareous marls. Generally, the habitats of *S. nigricans* are waterlogged, with the water table slightly below the surface. *S. nigricans* is normally pollinated by wind, but sometimes it also able to self-pollinate. Pollen is shed from early July onwards and seed is set in August. The seed is held firmly in the

spikelet, and may not be shed until December. This species is considered a typical pioneer species in wet dune slacks (Ernst & Van der Ham 1988). Estimations of the age of *S. nigricans* tussocks can be made by measuring the tussock diameter and number of inflorescences (Ernst & Van der Ham 1988).

Pedicularis palustris is limited to the northern hemisphere and occurs throughout Europe in natural fens and in moderately grazed or mown fen meadows. This hemi-parasitic species has a biennial life cycle and reproduces exclusively by seed. Seeds are buoyant and can be dispersed by water (*i.e.* hydrochory). During the first growing season, a rosette develops, which forms a winter bud in autumn (Ter Borg 1979). Flowering takes place in June and July of the second growing season. Flowers are pollinated by bumble bees (Kwak 1979). The species is short-lived and has only a short-term persistent seed bank (Thompson et al. 1997); thus population persistence of this species depends on regular reproduction by seed.

Study sites

The study was carried out in several dune slacks on the Dutch Wadden Sea island of Schiermonnikoog, the Netherlands (53°29' N, 6°12' E). Four sites were selected to study *S. nigricans*:

- Green beach - The youngest site for *S. nigricans* was *ca.* 15 years old, located on a beach plain and has never been managed;
- Kapenglop, eastern part - This part of the slack had been sod-cut in 1993, was thus 16 years old, and has not been mown since being sod-cut;
- Primary Valley - This site had an estimated age between 25 – 30 years old and has been mown regularly;
- Strandvlakte - The oldest site was 50 years old and has been mown recently.

Five sites were selected to study *P. palustris:*

- Kapenglop, middle part with both wet and dry sites - This was the youngest stage (5 years old) and had been sod-cut;
- Kapenglop, eastern part – This was the same site as used for *S. nigricans* (16 years old, no management);
- Primary Valley - This was the same site as used for *S. nigricans* (25 – 30 years old*,* mown regularly);
- Bernhard Valley – This was an old slack (ca. 100-200 years old) and its dry areas have been mown occasionally, whereas the wet areas have never been mown;
- Arnica Valley – This site had an estimated age of 400 years and has been mown annually.

Population structure

Studies on the population structure were carried out using 1 m^2 plots with four to five replicates for each species. The population structure of *S. nigricans* was described based on the tussock diameter class (<4 cm, 4 – 7 cm, 8 – 17 cm and >7 cm) and number of inflorescences, which was classified using a semi-log scale (see Ernst and van der Ham 1988). For *P. palustris,* individuals within the plots were classified using three life-history stages: seedling, juvenile and adult.

Soil analysis

Soils were sampled from 0 to 5 cm in depth in each plot using a 100 cm^3 metal ring. Samples were taken from a deeper layer if the organic layer was thicker than 5 cm. Samples then were homogenized after removing the vegetation and litter from the

surface. The homogenized samples then were divided into four portions. Three of them were used to determine organic matter, while the remaining portion was used for pH measurements. Organic matter content was determined after burning the soils at 550°C for at least for 15 hours. Fifteen grams of the remaining soil sample was uses for pH analysis. For pH analysis, the soil was added with 20 ml of distilled water to a test tube, left overnight to allow for exchange of anions and cations, and measured for pH. Another pH measurement, pH(KCl), was taken by adding 2.5 ml KCl 1N to the same samples since this value is more stable than pH(H_2O).

Seed viability experiments
To test seed viability of *S. nigricans*, seeds were collected from ten inflorescences per plot. For *P. palustris,* seeds were collected from one individual plant per plot. Fifty seeds were placed on moist filter paper and placed in a closed petri dish. Cold stratification (4°C) was applied to the seeds for six months (Oct 2009 – March 2010). The viability of the seeds was estimated based on the total number of germinated seeds.

Seed sowing experiment
The sowing experiment was only carried out for *P. palustris* in order to test the effect of inundation on the germination rate of this species. Seeds of *P. palustris* were collected from the population of Primary Valley in July 2010. Viability tests were conducted by germinating 50 seeds on wet filter paper in a closed petri dish using three replications. Seeds were treated using tetramethylthiramdisulphide (TMTD) prior to the test. The test was performed in a climate chamber with a light regime of 12 hours of light per day.

Seeds were sown on Schiermonnikoog in the dune slacks of Primary Valley, Kapenglop, Arnica Valley and Bernhard Valley; this species was found abundantly at all of these sites. Fifty seeds were put in a 5 cm^2 nylon-mesh bag (1 mm mesh size). Four seed bags were attached to metal pegs and were buried at a depth of about 2 cm below the surface. At each site, a set of seed bags was buried at different elevations (low, intermediate and high) for a total of fourteen bags per site. Seeds were left in the field from October 2010 until May 2011. After excavation, seeds were considered to have germinated if the cotyledon had appeared even if they had been found dead. The soil data and data from the germination experiments were analyzed using one-way ANOVA.

Results

Population structure
The population structure of *S. nigricans* in four different slacks (Figure. 1) showed that young sites (15 years and 16 years) had a higher frequency of seedlings compared to old sites. These two populations were characterized by an inverse J-shape, where young individuals were more numerous than mature individuals. In contrast, there was no significant difference between the three smallest classes of tussock diameter in the old sites. Additionally, the number of inflorescences was positively correlated with tussock diameter at all slacks (Figure. 2). This relationship however, was less clear at the oldest stage (50 years).

For *P. palustris*, most of the dry sites showed a higher number of seedlings (~300 individuals/m^2) compared to juvenile and adult stages. However, the number of

seedlings was very low in the dry sites of the 30-year-old and 200-year-old slacks (Figure. 3). Adults were the dominant life-history stage at all three of the wet sites.

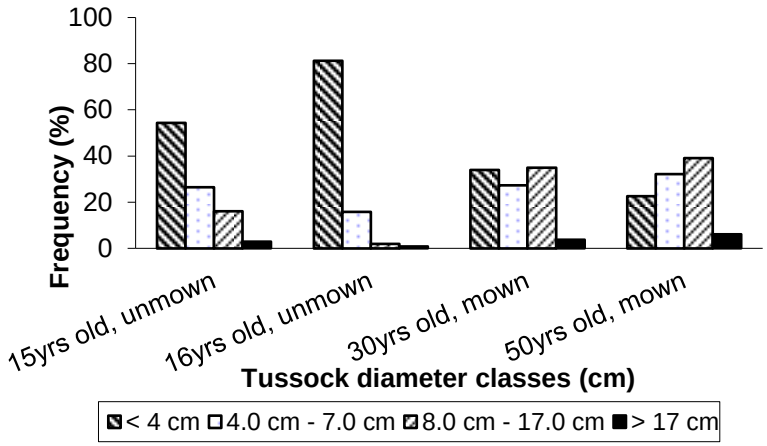

Figure 1. Population structure of *Schoenus nigricans* based on the frequency of individuals belonging to tussock diameter classes

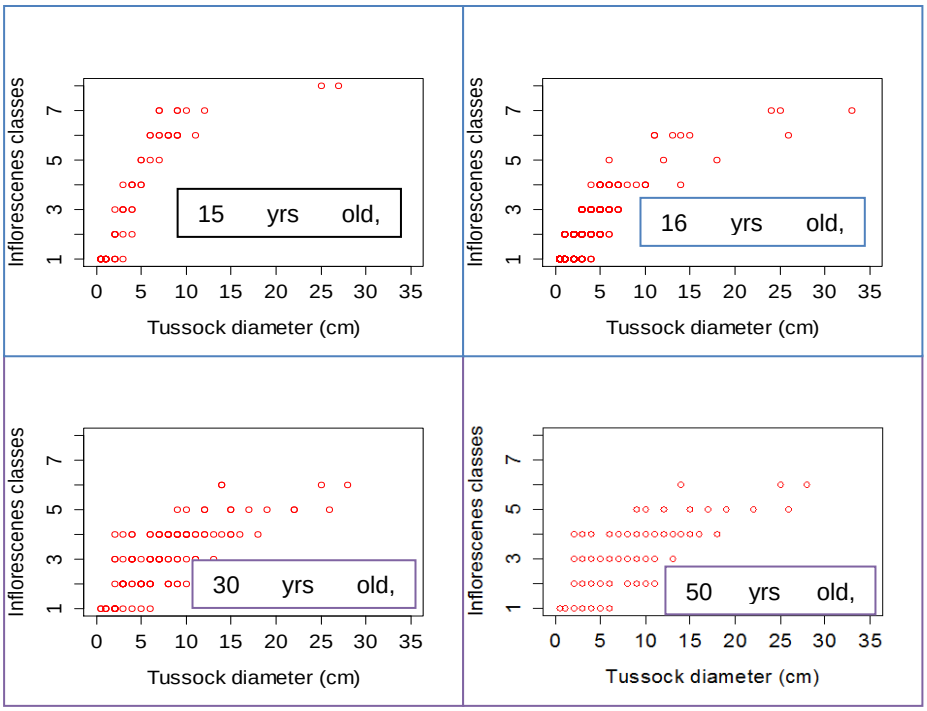

Figure 2. The relationship between tussock diameter and inflorescence class for *Schoenus nigricans*

The pH values were high (>6) for all studied sites; no significant differences were found between sites. However, the sites containing *P. palustris* showed quite a variation in organic matter content and pH (Figure 5). In general, wet areas accumulated slightly more organic matter than dry areas with the exception of the 5-year-old site, which had been recently sod-cut. It was also evident that organic matter content was linked to the age of the slack, *i.e.* old sites accumulated more organic

matter than young sites. No such clear trend was found for pH values at *P. palustris* sites. However, the wet areas of the 200-year-old site accumulated significantly higher organic matter than the dry areas.

Seed germination

In general, the germination tests for *S. nigricans* showed a low germinability (mean <14%) (Figure. 6a). Seeds from the Strandvlakte showed significantly higher germinability compared to those at other sites. The mean germination percentage for *P. palustris* was much higher (73%), but there were no significant differences between sites (Figure 6b).

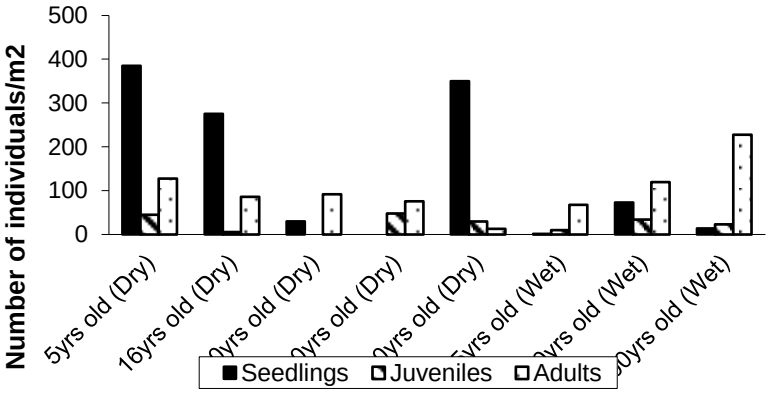

Figure 3. Population structure of *P. palustris*. Sites were arranged according to site condition (dry – wet) and slack age (young – old).

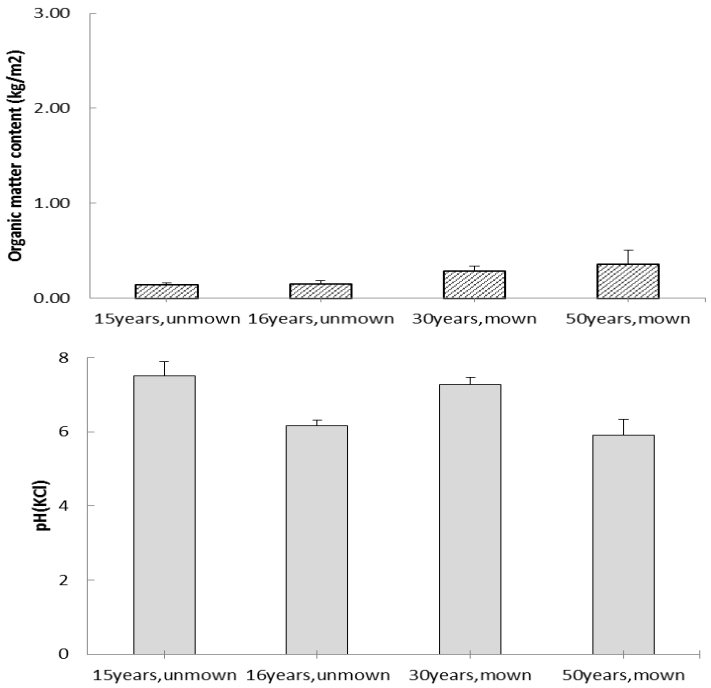

Figure 4. Soil organic matter content and pH for *S. nigricans* sites. Sites were arranged according to slack age (young – old) and management activities (unmown – mown).

Soil organic matter and pH

The soil organic matter accumulation was relatively low at the *S. nigricans* sites, with the highest accumulation found at the oldest site (50 years) (Figure. 4).

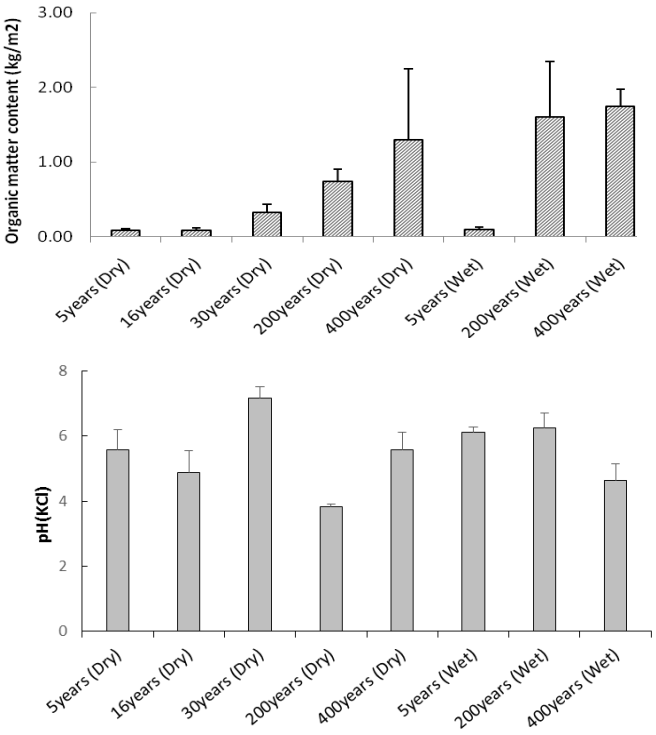

Figure 5. Soil organic matter content and pH for *P. palustris* sites. Sites were arranged according to slack age (young – old) and also site condition (dry – wet)

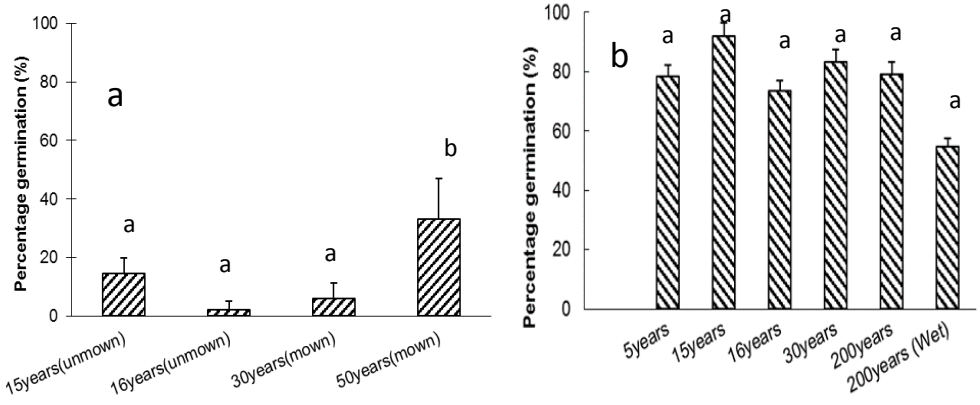

Figure 6. Percentage germination in laboratory trials of (a) *S. nigricans* seeds and (b) *P. palustris* seeds. Different lower-case letters indicate significantly different groups.

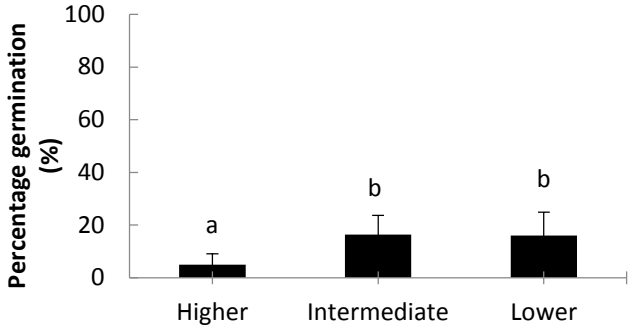

Figure 7. Percentage germination of *P. palustris* seeds, sown at three levels in elevation.

Discussion

The results of this study showed that the population structure of *S. nigricans* in younger slacks was characterized by a high number of seedlings, while older slacks were dominated by adults, which has also been reported by Ernst & van der Ham (1988). Our results also support the suggestion by Ernst & van der Ham (1988) that sod-cutting is an efficient management measure to restore *S. nigricans* vegetation. We found that the number of seedlings in the sod-cut site was much higher than at an unmanaged young site.

The organic matter content of soils at *S. nigricans* sites was relatively low (average 0.2 kg/m^2) and pH values were above 6.0 at all these sites. These high pH values are in line with the studies on *S. nigricans* in rich fens in other European countries (Wassen 1990, Beltman et al. 1996). An exception was found for Irish bog populations, which occurred at acidic sites (pH=4.0) (Boatman 1962, 1972). However, the same study found that seeds were incapable of germinating under these acidic conditions. The low germination rates found in the present study cannot be explained in this way, because all our sites had high pH values. The highest germination rates were actually found at the oldest site, which had more organic matter and slightly lower pH. This result was contrary to our expectations. We had expected that seeds from new populations and also from regenerated populations would be more viable than those from the old site, which would have been consistent with the results of Ernst & van der Ham (1988). Apparently, the oldest population in this study is still in good condition and produces seeds with relatively high viability compared to the new populations. This could possibly be explained by the differences between seeds that come from young and mature tussocks since the population of oldest site was dominated by mature tussocks (4 – 17cm).

In contrast, we did not find a clear relationship between population structure and slack age for *P. palustris.* Populations of *P. palustris* were found along a wide successional range varying from young slacks (5-10 years old) to a very old slack (~400 years old). Unlike *S. nigricans*, *P. palustris* is a short-lived species, thus no pattern could be found in their population structure that could be related to population age. However, we found very distinct differences in population structure between the dry and wet areas. High density of seedlings at the drier areas indicates that inundation is an influencing factor for seed germination in this species. The absence of seedlings in the population structure at the dry areas of Bernard Valley probably

can be explained by high acidity at this site (pH=3.8). It is possible that the soil pH could have been higher in previous years than what had been observed during period of sampling, which would have allowed seeds to germinate. This could be a reason why we still found juveniles and adults at this site. Alternatively, it is also possible that seeds had already germinated in the lower part of the valley, which is highly alkaline (pH> 6.0). The germinated seeds could then have been dispersed by water (during high water levels) from the lower part of the valley to the upper part and remained at this site when water levels dropped. Because this species is a hemi-parasitic plant, they would have been able to survive in acidic areas once they were attached to their hosts, which have larger and deeper root systems. A study on two *Pedicularis* species in the Czech Republic showed that this species did not have much affinity to any particular vegetation type (Petrů & Lepš 2000). This indicates that *Pedicularis* can survive under a wide range of environmental conditions, which supports the high variation in soil organic matter content and soil pH found in this study.

Even though there was no relation between organic matter and population structure, the increase of organic matter could have affected the population of *P. palustris* in the way explained by Fibich et al. (2010). Since this species depends on the hosts, the distribution, density and abundance of the hosts will affect the occurrence of *P. palustris*. *P. palustris* will decline when the aboveground biomass of the hosts greatly increases (see also Westburry & Dunnett 2007); this hemiparasitic plant will be outcompeted by the hosts' species. On the other hand, when productivity is too low (such as in the very early successional stage), *P. palustris* is also not able to survive due to the limitation of host availability.

The high percentage of germination in the seed viability experiment (laboratory conditions) for *P. palustris* might indicate that the viability of the seeds is not correlated with the physical environment. However, results from the seed sowing experiment (field conditions) indicate that *P. palustris* seeds germinate easily at the intermediate and lower elevation sites. A tentative conclusion can be made that seeds were not able to germinate in under three conditions: (i) when a site was inundated and seeds were completely saturated, (ii) when a site was too dry and, (iii) when seeds were floating. The first condition is supported by Ter Borg et al. (1985), who suggested that seeds will not germinate when under water. Furthermore, Petrů & Lepš (2000) found that high water levels during spring resulted in low germination of *P. sylvatica* in their experimental plots. A possible explanation for condition (ii) is that germinated seeds need moisture to establish. This is supported by a study on *P. furbishiae*, which showed that seedlings at drier sites showed higher mortality compared to those at wetter sites (Gawler et al. 1987). Probably in this study, the seed packets had been buried in sites that were totally dry, thus resulting in failed germination. This also evident when germinated seeds from lower elevation areas were observed to be more fresh compared to those found at intermediate sites, which had started to dry out. This indicates that this species needs wet conditions to survive directly after germinating. Once established, *P. palustris* is able to withstand a long period of flooding, which is shown by its presence in the wet sites in this study; some sites were completely inundated.

Chapter 5

Window of opportunity for *Liparis loeselii loeselii* populations during coastal dune-slack succession

Rohani Shahrudin, Annelies van de Craats, A. P. Grootjans and Annemieke Kooijman

Abstract

The rare orchid, *Liparis loeselii loeselii*, generally occurs in pioneer stages of calcareous dune slacks in coastal areas. The present study deals with the occurrence of *L. loeselii* populations in relation to successional stages and environmental conditions of dune slacks. The study was carried out in a series of natural and human-influenced dune slacks of different ages. Results showed that the window of opportunity for *L. loeselii* to colonize a new slack is relatively brief. The species was able to establish a population within the first 6 years after the first vegetation appeared. The population size increased the first 12 years to *ca.* 50 individuals/m^2 and then declined. Under favorable management measures, such as mowing, the population could survive at least for 20 years. We found that pH was significantly correlated with the occurrence of *L. loeselii* populations. Additionally, high Na/Ca ratios, NH4 and organic matter in the topsoil were correlated with sites where *L. loeselii had become extinct*. The results suggest that the optimal conditions for survival of *L. loeselii* populations during succession last for only a short time, even with the help of nature managers in older dune slacks. The populations of the Hors area on Texel, the Netherlands, could only survive for a longer period due to the continuous formation of new slacks in this area. Therefore, it is important not to interfere with natural processes that assist in the formation of new dune slacks.

Keywords: *landscape dynamics, pH*, *organic carbon, successional age*

Introduction

Liparis loeselii loeselii (L.) Rich. is a small orchid, which occurs in lowly productive, calcareous peatlands (fens) (Wheeler et al. 1998, McMaster 2001, Bednorz 2003, Rolfsmeier 2007, Pawlikowski 2008, Naczk & Minasiewicz 2010, Milanović 2012). It also occurs in coastal wetlands (interdunal slacks) on mineral soils (Jones & Etherington 1992, Lammerts & Grootjans 1998). *L. loeselii* can also be found in purely man-made habitats, such as gravel-pits (Bzdon & Ciosek 2006) and peat-excavated areas (Wheeler et al. 1998). *L. loeselii* has a wide distribution, ranging from the northeast of the United States and Canada, to most northern and Central European countries, Russia and even several localities in Siberia. Despite this wide distribution area, populations of *L. loeselii* are in serious decline along most of its geographical range. In Central Europe, for instance, it is now considered as an endangered species (Schnittler and Günther 1999) and has been listed in Annex II and Annex IV of the Council Directive 92/43/EEC on the Conservation of natural habitats, wild fauna and flora, thus making it a priority species for conservation in most European countries. Few demographic studies have been published on the ecology of this species (*e.g.* Jones 1998, Wheeler et al. 1998, McMaster 2001, Bednorz 2003).

The relationship between *L. loeselii* survival and successional stage in coastal wetlands in Wales was studied by Jones and Etherington (1992). They classified vegetation communities into five nodes that represented successional stages from open (pioneer) to late successional stages with high vegetation cover. They found that *L. loeselii* appeared between 12–15 years after initial vegetation of the slacks. The orchid started to decline when the shrub, *Salix repens,* developed a dense vegetation cover, which occurred after around 40 years. Several authors have indicated that *L. loeselii* populations cannot survive when the habitat is overgrown by woody species (Wheeler et al. 1998, McMaster 2001, Bednorz 2003, Bzdon and Ciosek 2006). Additionally, a dense cover of moss species such as *Calliergon cuspidatum* could also out compete *L. loeselii* (Jones and Etherington 1992); a thick moss layer could be a barrier for *L. loeselii*'s roots to access the underlying mineral soil. In addition to habitat age and vegetation factors, abiotic factors such as water level and pH, have also been mentioned in the literature to be important factors that influence the survival of this orchid. Jones (1998), for instance, reported the decline of this orchid during the low water levels of summer. Favorable conditions recorded for *L. loeselii* populations in fen habitats include a high pH (around 7) combined with low availability of nutrients in Eastern England (Wheeler 1998) and low amounts of organic matter on the Dutch and German Wadden sea islands (Petersen 2002, Davy et al. 2006). Stuckey (1967) showed a wider range of favorable pH values for fen populations in the United States (4.6 to 7.2). However, no study has thoroughly examined the relationship between other environmental factors and survival of *L. loeselii* populations during vegetation succession.

This study was conducted to investigate the time period that *L. loeselii* populations need to colonize new habitats and also how long a population can survive. The successional sequence of wet dune slacks on the southern tip of Texel, the Netherlands, is a good model system to study the occurrence of this orchid during different successional stages. The development of the dunes in this area has been well documented, which enabled us to accurately assess dune-slack age. Our general aim was to describe the survival rate of *L. loeselii* throughout the dune-slack successional stages and relate this survival rate with habitat parameters and management measures. We specifically addressed two questions: Firstly, what is the

length of the window of opportunity for *L. loeselii* on the southern tip of Texel? And secondly, which factors best predict the occurrence of *L. loeselii*?

Material and methods

Study areas

This study was conducted on the Dutch Wadden Sea Island of Texel. At the southern tip of the island, The Hors (52°59'N, 4°44'E), several populations of *L. loeselii* were present (Figure. 1). The Hors is a very young dune area with a broad beach plain. Relatively small, but very dynamic dunes have formed small dune ridges that prevent regular flooding of the dune slacks by the sea. Seven sites were selected in the Hors area. The selection was based on dune age and the occurrence of *L. loeselii* (Figure. 1). We included one site where *L. loeselii* had become extinct, five sites with extant populations and one very young slack where we expected the orchid to appear within a few years. The sites were: west of Hors lakes (has been mown since 1998), Grey Goose valley (has been mown since 1998); the eastern and central areas of the Kreeft polder (has been mown since 1998); Hors valley (no management); the westernmost part of The Hors (no management); and the southern part of the Hors (no management). Some of these dune slacks are anthropogenous in origin. In the mid-1970's, several sand dikes were developed artificially. These sand drift dikes now keep a part of the Hors's beach plain from the influence of the sea (the present Hors and Kreeft polders). The site where *L. loeselii* populations have become extinct, despite intensive management measures, is situated to the west of the Hors lakes.

Age estimation

In order to estimate the age of dunes and dune slacks in the Hors area, we used information from historical maps (Oost et al. 2004). Firstly, we distinguished slacks that had developed before 1986 from those that had formed after this year. From 1986 onwards, aerial photographs of Texel (year 1986) and aerial photographs of the Hors (year 1996, 2000, 2003 and 2008) were used to estimate the age of the slacks. We also used vegetation maps of the Hors area from various years (1986, 1988, 1994, 1996, 1999, 2004 and 2005), in order to interpret aerial photographs and to obtain information on the first colonization of *L. loeselii* populations in the slacks. Additional information on this point was obtained from field observations recorded by officials of the State Forestry Service (*Staatsbosbeheer*).

Data collection

Within each site, four to seven sub-plots with the size of 1 m^2 were sampled, depending on the size of the populations. We assessed the population structure of *L. loeselii* by classifying the individuals into three life stages: '1-leaf' for single-leaf juveniles, '2-leaf' for plants that had two leaves but no inflorescence, and 'adults' for plants that had flowered. The total number of *L. loeselii* individuals was also estimated at each site. Vegetation cover (%) in the sub-plots was estimated using the Braun-Blanquet approach. We also estimated vegetation height and percentage cover of shrubs, herbs, bare soil and mosses. Water levels and thickness of the organic layer were measured in bore holes. Soil samples were taken using metal rings (volume of 100 cm^3). The samples were pooled for a total volume of 200 cm^3 and transported to the laboratory prior to analysis.

Soil analysis

Samples were weighed before drying at 40°C-70°C to measure the water content. The dried samples were then homogenized, sieved through a 2-mm mesh and ground (5 minutes at 400 rpm) before drying (at 105°C for 24 hours). Total C and N

content were measured using a CNS elemental analyzer. Soil extracts were prepared by adding 100g of soil to 100g of distilled water. After shaking and centrifuging, the water extract was filtered using a 0.2 µm membrane filter. This final water extract was then used to analyze: carbon fractions with the TOC analyzer (Shimadzu TOC-Vcph); pH, Electrical Conductivity (EC), anion concentration (Cl^-, PO_4^{3-}, NO^{3-}, NO^{2-}, DOC, SO_4^{2-}, NH_4^+), cation concentration (Ca^{2+}, Na^+, K^+, Mg^{2+}, Al^{3+}, Fe, Mn, P, S, Si, Zn) and alkalinity using the Inductive Coupled Plasma Atomic Emission Spectrophotometer (ICP-AES).

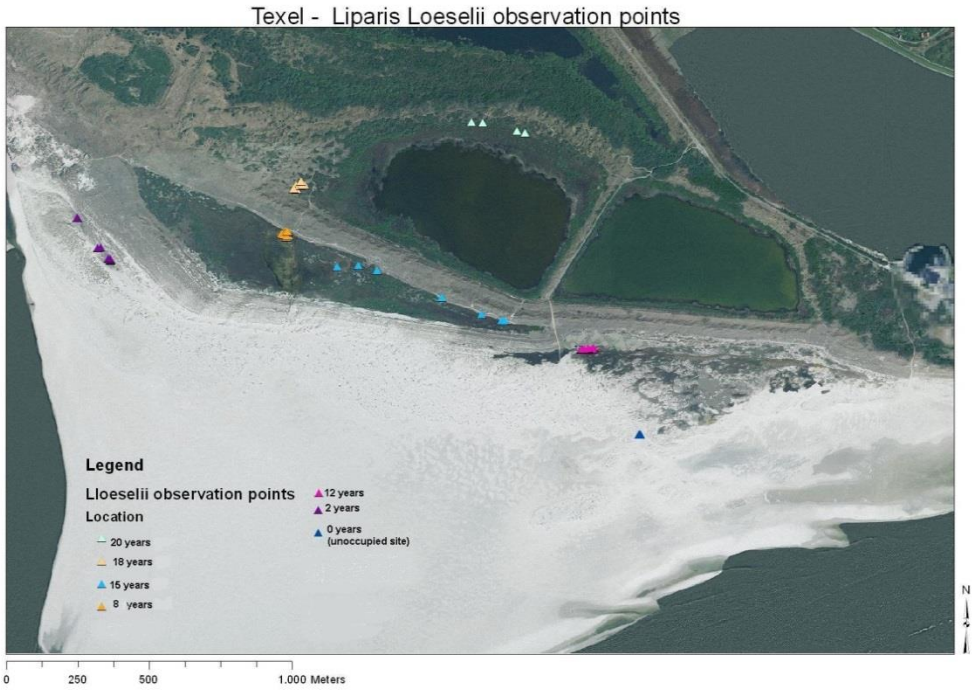

Figure 1. Map showing sampling locations on southern Texel.

Data analysis

In total, 53 variables were measured from the 7 sites, including parameters related to vegetation, fresh soil, those measured from the soil water extractions and water level. A Pearson correlation test was conducted to test for significant correlations between population age and these environmental parameters. *L. loeselii* populations were classified into 3 groups: (i) Extinct populations, (ii) Extant populations, and (iii) Predicted populations. Differences between groups with respect to the environmental parameters were tested using a One-way ANOVA, followed by a Bonferroni post-hoc test. For multivariate analyses, we used the program, CANOCO (Ter Braak & Smilauer 2002), in order to study relationships between vegetation composition, environmental variables and *L. loeselii* occurrence. Detrended Correspondence Analysis (DCA) was first applied to the species composition data. The data were square root transformed to minimize the effect of dominant species. Axes were detrended by segments and rare species, down-weighted. Based on the length of the gradients (>4.0) estimated by DCA, the unimodal constrained ordination of Canonical Correspondence Analysis (CCA) was selected to analyze differences in *L. loeselii* occurrence. In the CCA analysis, scaling was focused on inter-sample distance and bi-plot scaling was applied. In order to find significant discriminating variables, we applied forward selection to all variables followed by a Monte-Carlo permutation test (999 permutations with a significance level of 0.05).

Results

Dune-slack development and population establishment

The oldest site where *L. loeselii* has occurred is 34 years old (Table 1) but *L. loeselii* disappeared from this site about 12 years ago. The population age of this site before their disappearance was estimated to be at least 20 year old since the species was first spotted here in 1998.

The oldest estimated age for an extant population was 18 years, whereas the youngest population was estimated to be 2 years old. The time needed for *L. loeselii* to colonize a new dune slack varied between 2 to 25 years. Based on the results of the oldest slack, we estimated that the life span of *L. loeselii* populations in this area could reach at least 20 years.

The variables that are significantly correlated with population age are shown in Table 2. Positive correlations were found between population age and total vegetation cover, carbon (C), dissolved organic carbon (DOC), total organic carbon (TOC), total carbon (TC), sodium/calcium ratio (Na:Ca), potassium and aluminum, whereas negative correlations were found with percentage cover of bare sand, pH, alkalinity and silica (Table 2).

Table 1. Summary of sites used to estimate stage of dune slack development and age of *Liparis loeselii* populations

Location	Coordinate (Dutch grid)	Dune age according to historical map (years)#	Dune age according to OSL (years)#	Slack age according to aerial photographs (years)	*L. loeselii* population age in 2010 (years)
West of Hors lakes	111498 557847	57	69±4	NA	34*
Grey Goose valley	110906 557659	57	NA	24	18
East of Kreeften polder	111054 557381	31	NA	16	15
Hors Valley	111930 557107	46	24±3	12	12
Centre of Kreeften polder	110852 557460	31	26±1	16	8
New slack	110141 557493	10	20±2	7	2
Future slack	112110 556759	5	13±2	1	0

NA = not available; # from Ballarini et al. (2003) and Oost et al. (2003); * = the population of *L. loeselii* is extinct

The number of individuals and population structure of this orchid were found to vary between the different age stages (Figure. 2). Between 2- to 12-years in population age, the number of individuals in each population increased but this number decreased at later stages. In the youngest population, the number of 2-leaf individuals was about the same as the number of juveniles, whereas in the 12-year-old populations, the number of 2-leaf individuals was the highest. At later stages, the

numbers of adults and 2-leaf individuals fluctuated. Juveniles (1-leaf) had the lowest frequency at all stages.

Table 2. Partial correlation coefficients for variables that are significantly correlated with population age of *L. loeselii loeselii* ($P < 0.05$)

Environmental variable	Correlation coefficient
Total vegetation cover (%)	0.393
Bare sand cover (%)	-0.459
C	0.463
DOC (Dissolved organic carbon)	0.587
TOC (Total organic carbon)	0.523
TC (Total carbon)	0.509
pH(KCl)	-0.614
Alkalinity	-0.399
Na:Ca	0.474
K	0.555
Al	0.518
Si	-0.615

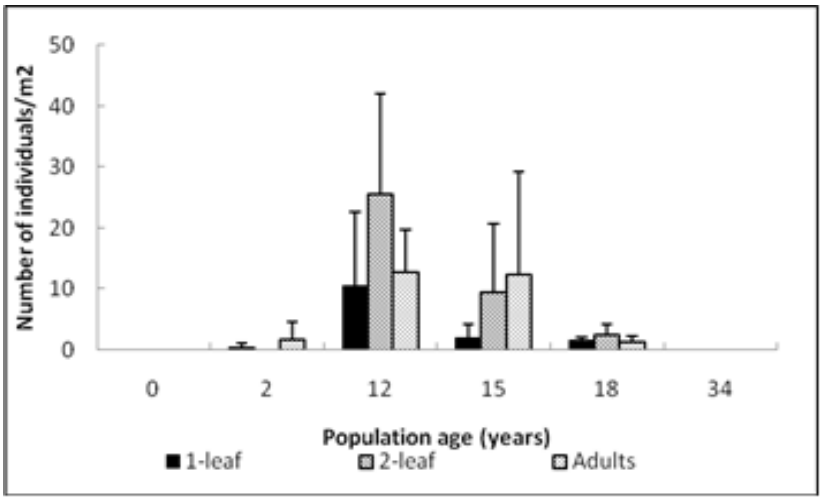

Figure 2. Population structure of *L. loeselii* according to age of population on the Hors, Texel. Error bars in the bar graph indicate standard deviation.

Environmental variables

Depth of organic matter (OM), carbon, pH and silica all differed significantly between sites with the three different types of populations (Extinct, Extant and Predicted; Table 3). Both thickness of the organic layer (from 4.3±2 cm to 10±2.1 cm) and the amount of carbon were higher in Extant than in Extinct sites.

The relationships between these three types of sites and environmental variables are shown in a CCA-biplot (Figure. 3). The first two axes of the CCA explained about 93 percent of the site variability with site-environment correlations of 0.98 and 0.95 for the first and second axes, respectively. Eigen values of the first two axes were 0.957 and 0.904, respectively. The percentage of bare sand was correlated with sites Predicted to form *L. loeselii* populations, whereas the Na:Ca ratio and NH4 were significantly correlated with sites from where *L. loeselii* had disappeared. The results

showed a positive trend between occurrence of *L. loeselii and* total vegetation cover, pH and silica but these relationships were not statistically significant.

Associated vegetation

A CCA analysis of species occurrence in the three types of sites distinguished by the status of *L. loeselii* populations (Figure. 4) showed which species were closely associated with the occurrence of *L. loeselii*. The first two axes explained 21.4 percent of the species variability, whereas the site-species correlations were 0.98 with the first and 0.90 with the second axes, respectively. Eigen values of the first two axes were 0.551 and 0.313.

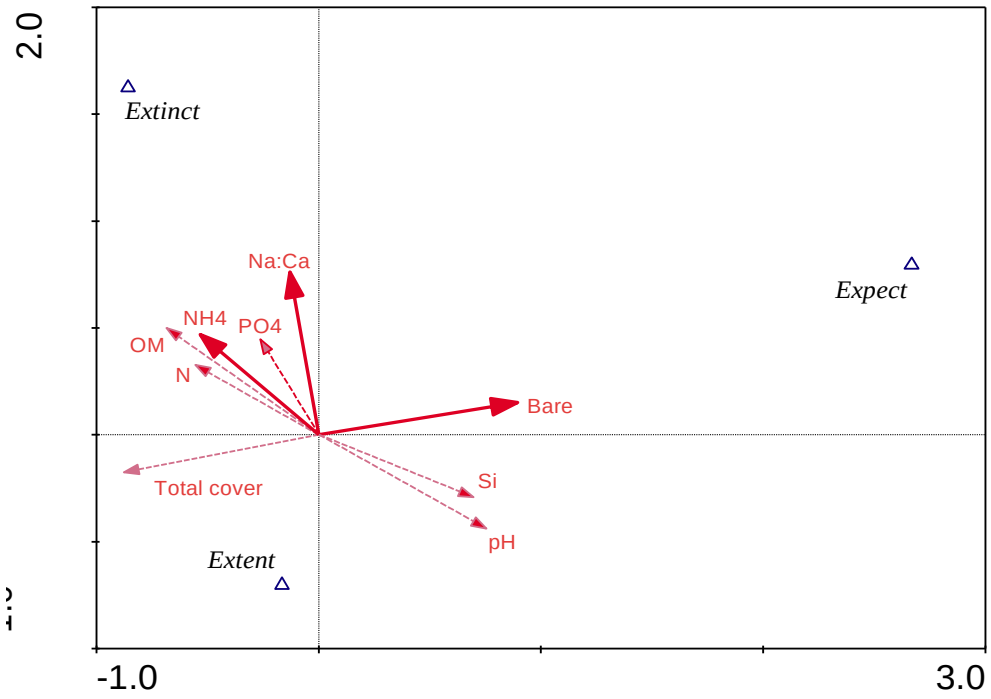

Figure 3. Canonical Correspondence Analysis (CCA)-biplot of sites and environmental variables. Bold arrows indicate the selected discriminating variables (Bare – percentage of bare sand, Na:Ca ratio and NH4). The selected variables were significant ($p<0.05$) (999 permutations of Monte Carlo permutation test). The directions of arrows indicate where variables had a stronger effect.

The Extinct sites were associated with species of wet grasslands such as *Prunus vulgaris*, *Holcus lanatus*, *Trifolium repens*, *Festuca ovina* and *Trifolium repens*. Sites where *L. loeselii loeselii* still occurred were characterized by typical dune-slack species such as *Carex oederi*, *Parnassia palustris, Epipactis palustris, Linum catharticum, Schoenus nigricans, Centaurium littorale* and *Pyrola rotundifolia.* Meanwhile, the Predicted sites on relatively bare beach plains were associated with species usually found in embryonic dunes, such as *Ammophila arenaria* and those indicating brackish conditions, such as *Glaux maritima.*

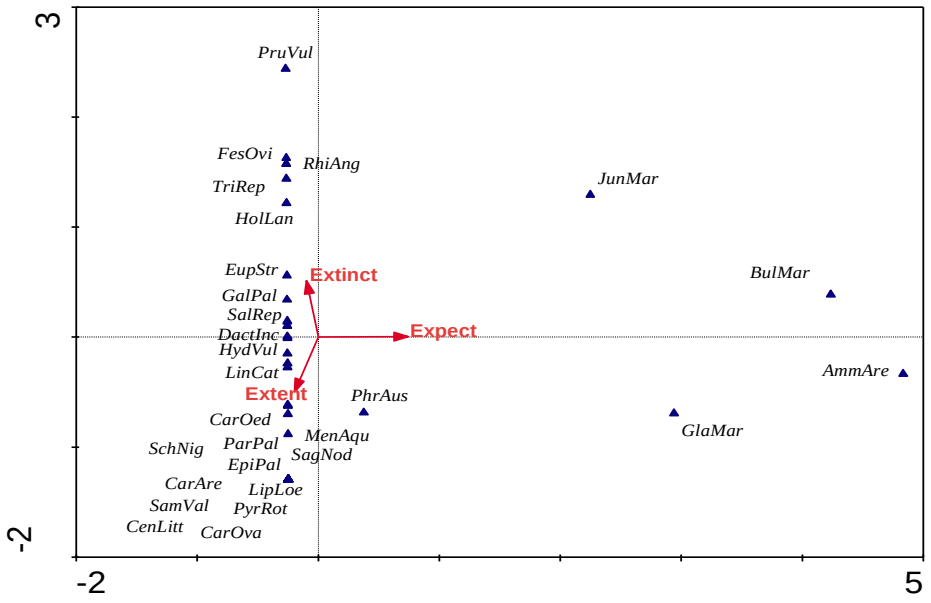

Figure 4. Canonical Correspondence Analysis (CCA) of vegetation community for sites with Predicted, Extant and Extinct populations of *L. loeselii*. (Abbreviations of species names: *JunMar=Juncus maritimus, BulMar=Bolboschoenus maritimus, AmmAre=Ammophila arenaria, GlaMar=Glaux maritima, PhrAus=Phragmites australis, MenAqu=Mentha aquatica, SagNod=Sagina nodusa, LipLoe=Liparis loeselii loeselii, PyrRot=Pyrola rotundifolia, CarOva=Carex ovata, CenLit=Centaurium littorale, SamVal=Samolus valerandii, CarAre=Carex arenaria, EpiPal=Epipactis palustris, ParPal=Parnassia palustris, SchNig=Schoenus nigricans, CarOed=Carex oederi, LinCat=Linum catharticum, HydVul=Hydrocotylae vulgaris, DacInc=Dactylorhiza incarnata, SalRep=Salix repens, GalPal=Galium palustre, EupStr=Euphrasia stricta, HolLan=Holcus lanatus, TriRep=Trifolium repens, FesOv= Festuca ovina, , RhiAng=Rhinanthus angustifolius, PruVul=Prunus vulgaris.*

Discussion

Dune-slack succession and window of opportunity for L. loeselii

In general, determining the age of coastal dune slacks can be difficult. However, we could make a reasonably accurate estimation of age in our study by using information from several sources, such as aerial photographs, historical maps, vegetation maps and field observations. We found that vegetation maps were more reliable in assessing dune-slack age than other method used, including historical maps and OSL measurements. The OSL technique to estimate dune ages corresponded well with information gleaned from historical maps (Ballarini et al. 2003), but since they were aimed primarily at gauging the age of the surrounding dunes, they tended to overestimate the dune-slack age. For example, recurrent episodes of sand blowing in the slack itself may start the succession anew. In fact, the OSL method gave a standard error of ±5 years, which is a significant error with respect to the temporal scale of dune-slack development.

Our results showed that the window of opportunity for *L. loeselii to establish a population* was relatively short, less than 20 years, but this species was able to take advantage of this short window. Populations of this orchid were able to colonize newly formed slacks at very early stages. Two years after a slack had become vegetated, *L. loeselii* was already able to establish a small population. Sometimes the orchid was able to establish its population even before the formation of a slack

was completely finished. This indicates that *L. loeselii* is a well-dispersed species, and can colonize a new site easily, as suggested by Jones (1998). A high number of individuals at the 2-leaf stage could be a positive indication for the populations in this study, as mortality was shown to be high at earlier stages (54% per year) for *L. loeselii* populations in the UK (Wheeler et al. 1998).

Table 3. Range, mean and standard deviation for the environmental variables measured on sites where *L. loeselii* used to occur (Extinct populations), occur now (Extant populations) and are expected to occur (Predicted populations). The variables were significantly different for these three site based on One-Way ANOVA ($p<0.05$)

	Extinct (n=4)	population	Extant (n=39)	population	Expected (n=4)	population
Variables	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD
Total vegetation cover (%)	80-100	96 ± 6.8^{a}	70-100	91 ± 11^{b}	20-50	$29\pm14^{b,c}$
Bare sand cover (%)	0-5	1.9 ± 2.6^{a}	0-30	8 ± 10.7^{a}	50-80	$71\pm14^{b,c}$
Moisture (%)	28.5-201	86.3 ± 57^{a}	9-216	$44\pm43^{a,c}$	5.5-18	$15\pm6.2^{b,c}$
Organic matter depth (OM) (cm)	13-Jul	10 ± 2.1^{a}	0-9	4.3 ± 2^{b}	0	0^{c}
Carbon (C)(mol)	53644-169427	100058 ± 37471^{a}	10768-125666	51853 ± 30671^{b}	6238-7821	6913 ± 764^{c}
Dissolved org. carbon (DOC) (mmol)	963-1810	14528 ± 355^{a}	186-1545	$675\pm316^{b,d}$	186-402	$307\pm101^{c,d}$
Total organic carbon (TOC) (mmol)	1080-1977	1550 ± 371^{a}	304-1400	$728\pm308^{b,d}$	161-344	$268\pm88^{c,d}$
Total carbon (TC) (mmol)	1081-1978	1551 ± 371^{a}	335-1430	$756\pm301^{b,d}$	213-424	$332\pm101^{c,d}$
Nitrogen (N) (mmol/m^2)	4232-5400	4624 ± 529^{a}	610-5796	$2879\pm1505.8^{b,d}$	219-423	$351\pm92^{c,d}$
Ammonium (NH_4) (mmol)	71.95-97.22	80.99 ± 11.77^{a}	7.46-81.98	$38.91\pm20.89^{b,d}$	2.19-17.06	$8.6\pm6.9^{c,d}$
Phosphorus (P) (mmol)	10.51-20.36	14.27 ± 4.27^{a}	1.16-21.17	$4.9\pm4.4^{b,d}$	1.37-3.43	$2.65\pm0.92^{c,d}$
Phosphate (PO_4) (mmol)	7.98-23.99	14.42 ± 6.92^{a}	0.32-24.98	$4.51\pm5.18^{b,c}$	1.29-3.45	$2.29\pm0.90^{a,c}$
pH(KCl)	4.2-6.1	5.4 ± 0.6^{a}	6.0-7.6	6.7 ± 0.4^{b}	8.0-8.4	8.1 ± 0.2^{c}
Sodium/Calcium ratio (Na:Ca)	4.32-7.58	5.68 ± 1.40^{a}	0.59-2.84	$1.49\pm0.60^{b,d}$	1.51-4.95	$2.60\pm1.62^{c,d}$
Potassium (K) (mmol)	38.05-79.96	60.67 ± 18.33^{a}	7.21-80.76	$32.18\pm16.52^{a,c}$	7.85-20.08	$16.34\pm5.71^{b,c}$
Aluminum (Al) (mmol)	0.75-1.83	1.34 ± 0.45^{a}	0.11-2.03	$0.62\pm0.33^{b,d}$	0.05-0.89	$0.39\pm0.38^{c,d}$
Manganese (Mn) (mmol)	0.08-0.30	0.18 ± 0.10^{a}	0.2-0.48	$0.09\pm0.09^{a,c}$	0.01-0.03	$0.02\pm0.01^{b,c}$
Silica (Si) (mmol)	2.56-4.92	4.02 ± 1.05^{a}	6.62-15.92	10.83 ± 2.2^{b}	13.59-20.39	16.97 ± 2.78^{c}
Zinc (Zn) (mmol)	0.03-0.05	0.04 ± 0.01^{a}	0.01-0.05	$0.02\pm0.01^{b,d}$	0.1-0.1	$0.01\pm0^{c,d}$

The short window of opportunity for *L. loeselii* during natural vegetation succession in the Hors area could only be extended modestly (*ca.* 5-10 years) by a management regime of annual mowing in the oldest dune slacks. In comparison, populations on a beach plain on the German Wadden Sea island of Borkum survived for more than 30 years without any management intervention (Petersen 2003). This species was first seen at this German site in 1985 and a population with many individuals still exists there today (3,000-10,000; Petersen 2010).

Key environmental factors for L. loeselii

Our study confirms that *L. loeselii* prefers young to medium or old vegetation stages and does not tolerate habitats with frequent flooding by seawater, as indicated by a

significant correlation with the Na/Ca ratio. We found a negative effect of soil organic matter on the survival of *L. loeselii* populations as has been reported by previous studies (*e.g.* Wotavová et al. 2004; Janečková et al. 2006). In our study area, the populations decreased or disappeared when organic matter increased. The accumulation of organic matter in the soil alters many soil properties, such as soil moisture and pH, and consequently also has a large impact on mineralization rates (Berendse et al. 1998).

The preference of this orchid for habitats with a high pH (>6) is consistent with results of other studies. Consequently, this orchid will head into decline when a dune slack starts to acidify. However, the ecological mechanism that leads to the decline of *L. loeselii* populations during acidification is still unknown. Dijk & Eck (1995), while studying another orchid species, *Dactylorhiza incarnata*, suggested that ammonium (NH_4^+) toxicity was responsible for the decline of that species. Van den Berg et al. (2005) reported a similar result for the decline of species sensitive to acidity, such as *Antennaria dioca* and *Succisa pratensis.* Our field study appears consistent with this hypothesis on ammonium toxicity for *L. loeselii* since NH_4^+ and pH were inversely correlated to each other in our CCA analysis. However, further study is needed to confirm this putative mechanism for the decline of *L. loeselii* after acidification.

Surprisingly, we did not find that water levels were important for the occurrence of *L. loeselii* populations in wet dune slacks, since various authors have suggested that disruption of the hydrology has a detrimental effect on *L. loeselii*. Water levels may be relevant in several ways. On one hand, this factor plays an important role in maintaining the calcareous environment and prevents the build-up of organic matter (Etherington 1975; Adema et al. 2005). Since pH is a decisive factor in influencing the occurrence of this orchid, it is important that *L. loeselii* sites are provided with sufficiently calcareous groundwater. Factors that lead to the low water levels, such as extracting water for drinking, ditching and afforestation, as well as longer dry periods will affect populations of *L. loeselii.* Low water levels promote desiccation, increased accumulation of organic matter and increased acidification, which in turn triggers the invasion of tall grasses or shrubs (Adema et al. 2005). On the other hand, a longer inundation period may hinder seed recruitment since mycelia of the mycorrhizal fungus that is associated with *L. loeselii* cannot grow under anaerobic conditions (Read et al. 1998). We probably did not find a negative effect of flooding because the summer of 2010 was not very wet. Additionally, as our dune slacks are still very young, decalcification may not yet have occurred and the soil itself is still buffered against acidification. This is probably the reason why low water levels were not yet crucial to regulating pH of the soil.

Conclusion and conservation implications

Overall, our results showed that populations of *L. loeselii* have a very short window of opportunity for existence in dune slack habitats. This window of opportunity opens after a newly formed slack starts to become vegetated, which allows for colonization by this orchid. This opening can occur sometime between one to six years after the first vegetation appears on the bare sand. The window of opportunity for a population can last up to 20 years and begins to close when soil pH decreases, followed by encroachment of light-competitive, taller species.

The information from this study is important when considering the fact that inland populations of this orchid are declining due to massive habitat alterations such as conversion of land for agriculture purposes. Coastal dune slacks nowadays receive much attention in conservation strategies (Davy et al. 2006) and dune slacks may

represent one of the last refuges for *L. loeselii* in Western Europe. The situation in the United Kingdom demonstrates the importance of dune slack habitats for the survival of this species. About 90% of the UK population is now only found in the coastal sand dunes of South Wales and population decline in this habitat is also evident (Jones et al. 1995). Dune stabilization was one of the factors that caused the decline of the UK populations (Jones et al. 1995). Since coastal sand dunes are a dynamic habitat compared to fen habitats, this indicates that the dynamic nature of these habitats is important for the survival of this species. The importance of dynamic processes in dune formation was also shown by Petersen (2010) who reported on stable populations on the island of Borkum (Germany) where populations were established 30 years ago and still survive to the present day. This is only possible when populations can easily move between old sites and new ones within a larger area undergoing dynamic dune forming processes. Therefore, it is very important not to interfere with or restrain the natural processes leading to the formation of new dune slack

Chapter 6

Genetic structure and dispersal events in a metapopulation of self-pollinating orchid in dynamic coastal wetlands

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Abstract

Species of highly dynamic habitats are expected to show high metapopulation dynamics due to frequent population turnover, which will subsequently affect genetic variation. We investigate the genetic structure of the self-fertilizing orchid *Liparis loeselii*, which occurs in the early to intermediate successional stages of coastal interdunal wetland (dune slacks). Using AFLP markers, we genotyped 581 individuals from 21 populations originating from six islands in the Wadden Sea. To detect the most likely source population for the colonizers, we used an assignment test on the most recently founded populations. Total genetic variation was rather high (H_j=0.30), with 51% of the variation residing within populations. Genetic differentiation among populations was also high (F_{ST}=0.49). The assignment tests revealed that most seed dispersal events occurred within islands. However, not all migrants came from the closest populations. These putative dispersal events were supported by the absence of an isolation-by-distance pattern, suggesting that the strong genetic structure did not result from limited seed dispersal. Our results suggest that breeding system and high turnover contributed to the genetic differentiation among populations. However, these processes are counteracted by high seed dispersal, which is able to maintain the high genetic variation within *L. loeselii* populations. For conservation of the species' genetic diversity, formation of new habitat patches should be promoted to maintain the metapopulation dynamics.

***Keywords**: AFLP, genetic differentiation, metapopulation dynamics, succession, selfing*

Introduction

Most populations do not form separate units, but are connected with others through dispersal. The resulting population networks are also known as metapopulations (Levins 1969) and form an important backbone of landscape ecology, genetics and conservation biology. The persistence of a metapopulation depends on the balance between local extinction and colonization (Hanski & Simberloff 1997), processes which consequently affect the metapopulation's genetic structure.

Studying the movement of migrants between populations is essential to understand the effects of colonization on metapopulation viability. Genetic studies of connectivity among populations have provided much insights into plant metapopulation dynamics (Giles & Goudet 1997; Hastings & Harrison 1994; Tero *et al.* 2003; Jacquemyn *et al.* 2004; Jacquemyn *et al.* 2006a; Honnay *et al.* 2009; Barluenga *et al.* 2011). Genetic approaches can be highly effective for detecting long distance dispersal. Assessing genetic differentiation among populations (F_{ST}) (Wright 1949) and also assignment tests (Paetkau *et al.*1995) are effective tools for estimating and specifying migration patterns (Cain *et al.* 2000; but see Whitlock & McCauley 1999).

A growing body of research has linked the effects of metapopulation dynamics on the population genetic structure (e.g. Giles & Goudet 1997; Jacquemyn *et al.* 2004, 2006a; Honnay *et al.* 2010; Vandepitte *et al.* 2007). For example, newly founded populations were more differentiated than intermediately aged populations of *Silene dioica* in a Swedish archipelago (Giles & Goudet 1997), indicating significant founder effects. Together with other bottlenecks associated with colonization, founder events are known to reduce genetic diversity (Pannell & Charlesworth 1999). Extinction-colonization dynamics lower the effective population size (Ne), thus increasing the chance of random loss of genetic variation (Wang & Caballero 1999). Therefore, species that occupy dynamic ecosystems, in which extinction and colonization is common, are expected to exhibit low genetic diversity and high genetic differentiation. However, there was no evidence of declining genetic diversity in the metapopulation of *Erysimum cheiranthoides* following several extinction and colonization events along the Meuse River (Honnay *et al.* 2009), although genetic differentiation increased. The effects of metapopulation dynamics on genetic variation also depend on the mating system. For example, theoretical models showed that the decrease in genetic variation due to colonization and extinction processes was stronger in self-pollinating species (Maruyama & Tachida 1992; Ingvarsson 2002).

Coastal wet dune slacks provide a mosaic of habitat patches that are ideal for studying the effects of recurrent habitat formation and degradation on genetic variation. Succession in newly formed dune slacks starts with the colonization of bare sand by plant species adapted to very low nutrient availability. After about 5 years, this stage shifts to an intermediate successional stage in which many plant species occur (Lammerts & Grootjans 1998; Grootjans *et al.* 2004). After 10-15 years, when soil organic matter accumulates, slacks will be invaded by tall grasses and shrubs. This will lead to the decline of pioneer species especially when the soil pH decreases (Grootjans *et al.* 2002). For species depending on base-rich conditions, therefore, the habitat is suitable only for a relatively short time. Since there are few coastal areas with natural formation of new dune slacks, and many dune complexes have been degraded by water extraction and eutrophication (Grootjans 1988, Grootjans *et al.* 2002), many of the pioneer species associated with base-rich wet dune slacks are rare and have been placed on national and international red lists.

Here, we investigate the genetic structure and migration events in a large metapopulation of one of these pioneer species, the orchid *L. loeselii,* in order to elucidate the processes that affect the distribution of genetic diversity in its highly dynamic habitat. Because the species has an autonomously selfing breeding system (Catling 1980, Claessens & Kleynen 2011), pollen exchange among individuals and populations will be very rare, increasing the likelihood of genetic differentiation. However, as a typical orchid, this species produces dust seeds (Arditti & Ghani 2000). Therefore, at least occasional long distance seed dispersal is expected in *L. loeselii,* which will counteract the limited pollen exchange due to the autonomous selfing. We address the following questions:

(1) Is there a high level of genetic differentiation due to the limited pollen dispersal associated with self-pollination?
(2) Is there evidence of long-distance (i.e. among-island) seed dispersal within the *L. loeselii* metapopulation?
(3) What is the origin of the individuals in the most recently colonized dune slacks?
(4) Are older and larger populations genetically more diverse?
(5) Is there a correlation between genetic and geographical distance?

Methods

Species

Liparis loeselii (Fen orchid) is an inconspicuous, short-lived, perennial orchid occurring in Europe and North-East America. The species is found in fens and dune slacks, particularly in the early stages of vegetation succession, on nutrient-poor and base-rich substrate. From the pseudobulb, a new shoot emerges at the end of May to mid-June after winter survival. Flowering occurs from late June to mid-July. The yellow-green flowers are small and hardly visited by insects (Claessens & Kleynen 2011). Self-pollination is assisted by rain droplets (Catling 1980). The pollinia fall onto the stigma spontaneously, resulting in autonomous self-pollination and high (i.e. nearly 100%) fruit set. The capsule reportedly produces a mean of 4,270 dust seeds (McMaster 2001). Seed viability is high, about 80% in one natural population in the USA (McMaster 2001). Over the last century, the species has strongly declined throughout its entire European distribution, and is therefore listed as endangered in both the EU-Habitat Directive and the Bern Convention with the highest conservation priority. In the Netherlands, a relatively large number of populations remain, so that the country has a special responsibility for conservation of this species.

Sampling

The study was conducted in the dune slack areas of the Dutch and German Wadden Sea islands. We collected samples from almost all known populations of the Dutch and German Wadden Sea islands. Borkum is the only German island harbouring *L. loeselii* (Petersen 2010). All *L. loeselii* sites are protected nature reserves. Most sites are not managed, but some sites, usually the older ones, are regularly mown.
Leaf samples from 596 individuals were collected from 28 populations from June to August 2010. We collected on average 24 individuals per population, but fewer samples were taken in small populations (Table 1). To avoid compromising their survival, only a small amount of leaf tissue (ca. 2-3 cm^2) was sampled, and only from adult individuals. Care was taken not to sample more than one leaf from groups of stems that potentially belonged to the same genet. The leaf samples were preserved in zip-lock bags with silica gel prior to DNA analysis. Population sizes were

determined by estimating the number of 2-leaved individuals at each site. Information on population age was obtained from *L. loeselii* observations collected by reserve managers or citizen scientists.

AFLP analyses

For genotyping, we used Amplified Fragment Length Polymorphisms (AFLP, Vos *et al.*1995) since this requires no prior knowledge of the *genome*. Furthermore, only a small amount of sampled tissue is needed for the DNA extraction, which is desirable when dealing with a rare species like *L. loeselii*. DNA was extracted using the CTAB method (Doyle & Doyle 1987) with minor modifications to suit the material. Before extraction, the dried leaves were homogenized into fine powder for 20 seconds in the Precellys®24 tissue homogenizer. After extraction, we inspected the quality of DNA on 1.5% agarose gel and its concentration and purity were measured using the Isogen Nanodrop ND-1000 spectrophotometer and subsequently standardized to 25 ng/µl. The AFLP followed the protocol from Vos *et al.* (1995) with minor modifications. AFLP analysis involves four steps: restriction, ligation, pre-amplification and end-amplification. The restriction and ligation was performed in a single step using 5µl of the 25 ng/µl DNA template. Pre-amplification of the fragments was performed by adding the restriction/ligation product (2x diluted) to the pre-amplification mixture with Eco-A primer and Mse-C primer (10µM; Biolegio, Nijmegen). The pre-amplification product was diluted 50 times prior to end-amplification. Two primer combinations were used in the end-amplification step: E-AGC/M-CTG and E-ACC/M-CTG (Applied Biosystems).

The amplification products were visualized on an ABI 3130 automated capillary DNA sequencer (Applied Biosystems). HiDi formamide (10 µl) was mixed with 0.25 µl Genescan LIZ®-500 internal size standard (Applied Biosystems, Nieuwerkerk aan den IJssel) and the mixture was spun briefly. The mixture of HiDi formamide/ LIZ (10 µl) was dispensed on the ABI 3130 titer plate and 1µl of the end-amplification product was added in each well. The samples were denatured at 94°C for 60 seconds before loading on the ABI 3130. The resulting AFLP electropherograms were analyzed using GeneMapper® software version 4.1 (Applied Biosystems, 2010). In order to calculate the error rates (Bonin *et al.* 2004), we replicated 60 samples that randomly selected from the DNA stock. The steps that involved in the replication were restriction-ligation, pre-amplification and end-amplification. The results from the duplicated samples were compared and mean of error rate was calculated as ratio of the number of mismatched loci to the total number of replicated loci. We found an error rate of 5.5%, meaning that 94.5% of the bands were found identical. Rare and common loci at a threshold of 10% were excluded to prevent statistical bias. In total, the two primer combinations resulted in 162 loci that were successfully scored for 596 samples. To assess any potential bias due to size homoplasy, we tested the correlation between fragment size and diversity of loci (Vekemens *et al.* 2002). This indeed showed a significant negative correlation between fragment size and frequency. However, it was not possible to remove this correlation by using a subset of the data based on only the larger fragments. Therefore the reported level of genetic differentiation is likely to be underestimated.

Data analyses

To quantify genetic diversity, we calculated allele frequencies using AFLP-Surv (Vekemans *et al.* 2002), using a Bayesian method with a non-uniform prior distribution of allele frequencies (Zhivotovsky 1999). With this method, it is possible to allow for self-fertilization; and we set the inbreeding coefficient to 0.95. Gene diversity, or expected heterozygosity (H_J), was computed for each population and

also for the whole metapopulation (H_T) following the method of Lynch and Milligan (1994). Linear regression was performed using R for Windows 2.11 (R development core team 2010) to test the relationship between gene diversity (H_J) and population size (N) and also between gene diversity and population age. We used an Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*1992) to partition total genetic diversity into within and among population components using GenAlEx version 6.5 (Peakall and Smouse 2006, 2012). Tests of significance were performed using 999 permutations. For all analyses, we only included the populations with at least 5 individuals, e.g. 21 of the 28 sampled populations.

We distinguished between the effects of isolation by distance and hierarchical population structure using a series of Mantel tests as outlined by Meirmans (2012a). We used GenoDive version 2.0b23 (Meirmans & Van Tienderen 2004) to calculate a matrix of pairwise F_{ST} values between pairs of populations. We also calculated a matrix of pairwise geographical distances and a model matrix representing whether pairs of population come from the same island or not. No transformation was performed on any of the matrices, since the fit was best when using the untransformed distances. We tested the association between genetic and geographical distance, taking the clustering within islands into account, with a stratified Mantel test in which populations were randomized within islands (Meirmans 2012a). We then tested for genetic differentiation among islands, taking the geographical distances into account, using a partial Mantel test (Smouse *et al.* 1986) between the matrix of genetic distance and the model matrix, using the matrix of geographical distances as a covariate. In addition, we used a standard Mantel test between matrices of among-island geographic and genetic distances (calculated as F_{CT} from an AMOVA). All calculations were performed in GenoDive and tests were performed with 9999 random permutations.

Principal Component Analyses (PCA) were performed in GenoDive (Meirmans & Van Tienderen 2004) based on a covariance matrix calculated from the allele frequencies of individuals and also of populations. For the PCA based on population allele frequencies, we also performed a significance test for the population differentiation represented by the PCA-axes (Goudet 1999). A clustering analysis was done using K-means clustering in GenoDive to determine the number of clusters in the data. This clustering method is based on the calculation of among-population Sums of Squares, equivalent to those used in an AMOVA (Meirmans 2012b). We used the simulated annealing option with 50000 steps and 200 random starts. We set the analysis to run from 2 to 20 clusters and the optimal number of clusters was chosen based on the Akaike Information Criterion (AIC), which was the only optimality criterion that provided a clear optimum number of clusters (we also tried BIC and pseudo-F, see Meirmans 2012b).
Assignments tests were carried out to detect colonization events into the most recently colonized populations but also to detect long-distance dispersal. This likelihood-based analysis uses the variation in allele frequencies among populations to assign individuals to their most likely population of origin (Peatkau *et al.* 1995). For detecting the origin of colonization, we selected per island one or two populations that established in the last 5 years. We then performed an assignment test on all individuals from that population against all other populations that were present at the time of colonization. The most likely source population was determined using the maximum likelihood $L_{Maximum}$. For detecting dispersal events, individuals were assigned to their most likely source population according to the likelihood ratio: L_{Home} / $L_{Maximum}$. To assess the significance of those assignments, we used the Monte Carlo approach of Paetkau *et al*. (2004) by randomly constructing new multilocus

genotypes by drawing multilocus gametes from the observed genotypes. For the Monte Carlo simulations we compared the results obtained when assuming full outcrossing with the results obtained assuming a selfing rate of 0.95. Furthermore we compared the results from using a very stringent significance threshold (α=0.002 as suggested by Paetkau *et al.* 2004), with those using a less stringent threshold (α=0.01).

Table 1. Populations of *Liparis loeselii* in the Dutch and German Wadden Sea islands. The only German island is Borkum, while other islands were the Dutch islands. Abbreviations: N, population size; n, number of sampled individuals; Age, estimated population age in years and H_J, expected heterozygosity (Nei's gene diversity)

Island	Population	Population code	Coordinate X	Coordinate Y	Age	N	n	H_J
Ameland	Ameland 1	Ameland 1	53.465769	5.907558	25	210	59	0.17
	Ameland 2	Ameland 2	53.465006	5.918547	35	34	21	0.12
Borkum	Borkum 1	Borkum 1	53.60984	6.759548	25	2980	12	0.12
	Borkum 2	Borkum 2	53.61163	6.745292	1	410	12	0.11
Schiermonnikoog	Groene Strand 1	Schier 1	53.498514	6.171256	3	300	30	0.27
	Kapenglop 1	Schier 2	53.491314	6.158439	5	4	4	-
	Kapenglop 6	Schier 3	53.490982	6.155296	5	20	12	0.08
	Kapenglop 8	Schier 4	53.491647	6.157654	5	1	1	-
	Groene Strand 2	Schier 5	53.498781	6.198378	2	300	31	0.14
	Stuifdijk	Schier 6	53.497708	6.21425	20	6	3	-
	Primary slack 1	Schier 7	53.487528	6.143136	5	300	30	0.11
	Primary slack 2	Schier 8	53.488251	6.144599	10	300	3	-
Terschelling	Boschplaat 1	Tersch 1	53.426911	5.415614	5	2	2	-
	Boschplaat 2	Tersch 2	53.428781	5.423392	5	1	1	-
	Noordsvaarder 1	Tersch 3	53.362525	5.177608	15	670	30	0.15
	Noordsvaarder 2	Tersch 4	53.375656	5.184953	14	770	30	0.15
Texel	Buiten Muy	Texel 1	53.119408	4.777195	33	9	1	-
	Ceres	Texel 2	53.029808	4.816533	8	25	12	0.21
	Secondary slack	Texel 3	52.998614	4.748589	12	200	32	0.18
	Goose slack	Texel 4	53.003981	4.730383	18	230	35	0.22
	Kreeftenpolder	Texel 5	53.001056	4.732697	14	1350	33	0.24
	Kreeftenpolder south	Texel 6	52.999822	4.743978	12	1350	30	0.2
	De Lange Dam	Texel 7	53.155953	4.816075	28	160	30	0.21
	Young primary slack	Texel 8	53.001311	4.719564	2	460	31	0.18
	Salty slack	Texel 9	52.997361	4.753047	10	200	32	0.25
Vlieland	First polder	Vlieland 1	53.257772	4.948775	38	230	30	0.14
	Fourth polder	Vlieland 2	53.253494	4.957219	20	16	16	0.12
	Military dune	Vlieland 3	53.248719	4.939647	5 - 10	66	33	0.12

Results

Genetic diversity

No significant relationship was found between the estimated population size and expected heterozygosity (r^2 = 0.00087; F = 0.0191, df 1 and 22, p-value: 0.89). There was also no significant relationship between population age and expected heterozygosity (r^2 = 0.00051, F = 0.0113, df 1 and 22, p-value: 0.91). The highest level of within-population diversity (0.27) was observed in a three year old population

on Schiermonnikoog (Schier 1); the lowest value (0.08) was found in a five year old population from the same island (Schier 3). At the island level, the highest genetic diversity occurred on Texel. Total population gene diversity (H_T) was 0.30, and the mean expected heterozygosity (H_J) was 0.17 (Table 1).

Population structure

The hierarchical AMOVA showed that a large proportion of the total genetic variation occurred within populations (Table 2). There was a strongly significant differentiation among islands (F_{RT} = 0.282, P=0.001) and among populations within islands (F_{SR} = 0.284, P=0.001); the overall level of genetic differentiation (F_{ST}) was 0.49 (P=0.001; 999 permutations).

Table 2. Analysis of molecular variance (AMOVA) for *Liparis loeselii* populations nested within islands. Only populations with at least 5 individuals were included in the analysis.

Source of variation	*df*	Sum of squares	Estimated variance	Percentage of variation	*F*	*P*
Among islands	5	4036.62	7.288	28	0.282	0.001
Among populations	15	2316.95	5.262	20	0.284	0.001
Within populations	560	7437.83	13.282	51	0.486	0.001
Total	580	13791.4	25.832	100		

Table 3. Results of standard, stratified and partial Mantel tests for distinguishing the effects of island-level clustering of populations and geographical distance.

Matrix A	Matrix B	Adjustment	Mantel's *r*	P-value
F_{ST}	Geographical among populations	–	0.252	0.0004
F_{ST}	Geographical among populations	Stratified: permuted within islands	0.252	0.94
F_{ST}	Model matrix of island sharing	Partial: geography as covariate	0.492	0.0001
F_{CT}	Geographical among islands	–	-0.238	0.22

With a standard Mantel test, a strong association was found between geographical and genetic distance (Mantel's r =0.252, p = 0.0004; Table 3). However, after a series of Mantel tests with several adjustments, we found that this association is not due to pure isolation-by-distance (IBD), but rather a side-effect of the genetic differentiation among the islands. The IBD pattern was no longer significant after correcting for the hierarchical population structure in a stratified Mantel test (Mantel's r =0.252, p = 0.94). Conversely, the differentiation among the islands remained significant after correcting for the influence of geographic distance (Mantel's r =0.492, p = 0.0001). When testing the pairwise F_{CT} differentiation among whole islands, there was also no association with the distance among islands (Mantel's r =-0.238, p = 0.22). These results indicate that the higher-order population structure is more dominated by differentiation among the islands than by limited dispersal.

The PCA also shows that there is generally a lack of clear geographical structure. Even though individuals generally clustered together per island (Figure1 – top), there are many exceptions to this and the locations of the different island clusters in the plot do not resemble the geographic proximity of the islands.

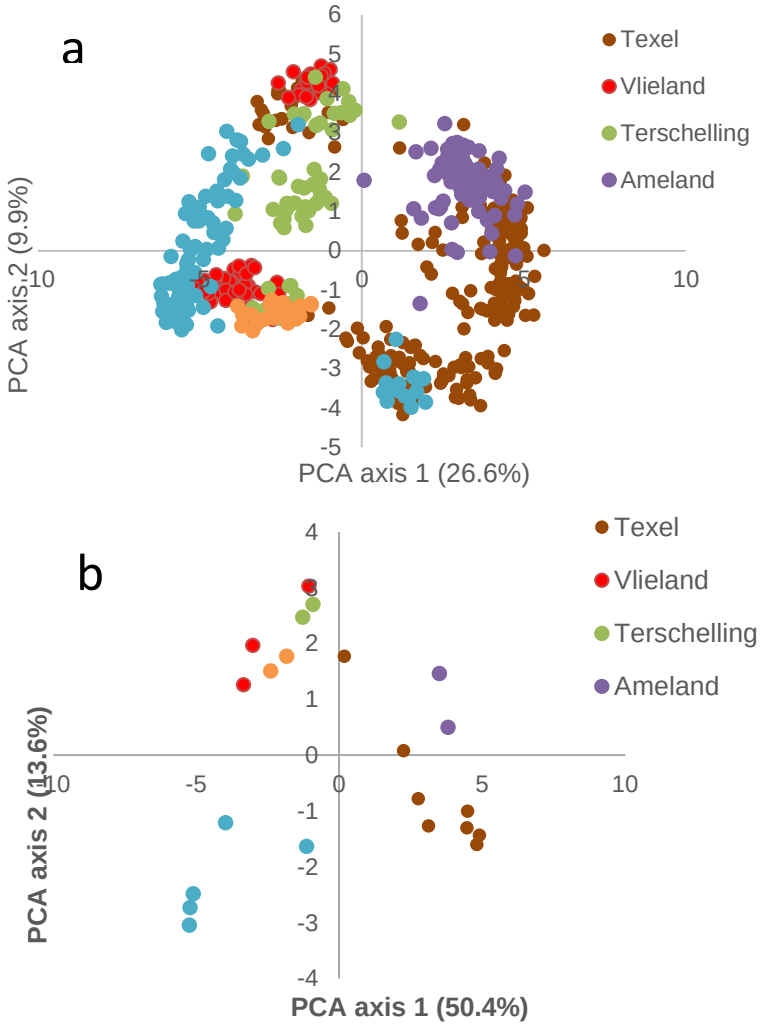

Figure 1. Principal Component Analysis of 581 individuals (a) and for 21 populations (b) of *L. loeselii*. For populations analysis, genetic differentiation was found highly significant on the axis 1 (F_{ST}=0.24, P=0.003), while axis 2 shows no significant difference (F_{ST}=0.065, P=0.62).

For example, the first PCA axis (explaining 26.6% of the variation) shows a clear clustering of the individuals from Ameland with most of the samples from Texel, even though these two islands are geographically separated by Vlieland and Terschelling. When the PCA was performed on the population level, populations from the same island generally clustered together, with Vlieland being the main exception. The permutation test showed that only the first PCA axis represents a significant (P=0.011) part of the among population variation (50.4%), corresponding to an F_{ST} value of 0.25.

K-means clustering using the Akaike Information Criterion (AIC) suggested 8 clusters. Populations from Ameland and Schiermonnikoog both formed their own cluster, while some populations on Vlieland and Terschelling were genetically similar to populations on Borkum and Texel (Figure2). Texel showed a patchwork of four different clusters, of which three were confined to Texel and the fourth was shared with Vlieland and Terschelling.

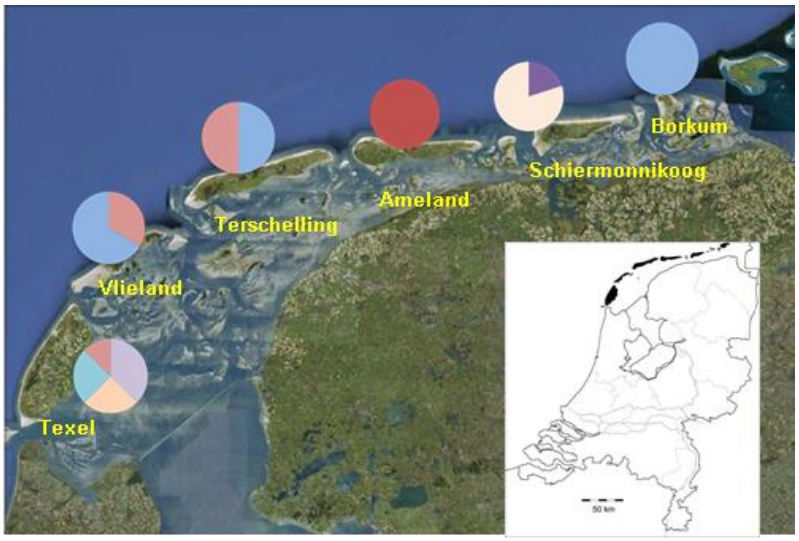

Figure 2. Map of the Wadden island chain with all *Liparis loeselii* populations in red dots. Pie charts indicate the proportion membership of individuals to the K-means clusters (k=8) for populations on each island. Inset shows the position of the Wadden islands in the Netherlands.

Migration events

When the assignment test was applied to the whole dataset, in order to detect recent migrants, the results strongly depended on the used approach. When self-pollination was assumed for the Monte-Carlo simulations (selfing rate=0.95), three individuals were tagged as migrants, compared to 43 when outcrossing was assumed (selfing rate=0). When a more stringent α-level (0.002) was used, no migration was detected when self-pollination was assumed. When only recently colonized populations were analyzed, the results revealed that 88% of all individuals (97 out of 110) were assigned to a population from the same island, while 12% came from a different island (Table 4).

Table 4. Assignment of individuals from newly colonized populations to putative sources. Migrants that travelled less than 5 km are in bold.

Source	Bork 1	Schier 1	Schier 3	Schier 7	Ters 3	Ters 4	Tex 4	Tex 5	Tex 6	Tex 7	Vlie 2	Vlie 3
Bork 2	**8**					2					1	1
Schier 5		**5**	**1**	**22**	1		2					
Ters 1					1	1						
Ters 2					1							
Tex 8								**5**	**25**	1		
Vlie 3	6										**27**	

The average dispersal distance was 16.2 km, though this is skewed by a relatively small number of putative long-distance dispersers: 85% of the colonizers (93 out of 110) had dispersed less than 4 km. Even though most of the colonization took place within the same island, the putative source population was not always the nearest population. For example, population Schier 5 was mostly colonized from Schier 7 (3.8 km), even though population Schier 1 is closer (2.2 km) and equally large. The longest distance was shown in the colonization of Vlieland 3, for which 6 individuals were assigned to population Borkum 1, which is 127 km away.

Discussion

As expected given its breeding system, *L. loeselii* showed a high level of genetic differentiation between all studied populations (F_{ST} = 0.49). Highly self-pollinating species normally exhibit strong genetic differentiation (Hamrick & Godt 1996). For example, strong genetic differentiation was shown in another study on a predominantly self-pollinating orchid, *Isotria medeoloides* (Stone *et al.* 2012). For *L. loeselii*, also Pillon *et al.* (2007) found a strong overall population differentiation (F_{ST} = 0.382) and an equally strong differentiation between populations from dune slacks and populations from fen habitats (F_{ST} = 0.370). However, whereas we found strong differentiation between islands, they did not observe any differentiation among populations from Britain and France. This difference may stem from the fact that they analyzed the fen and dune populations together, while we only focused on populations from dune slacks. These two habitats differ quite strongly in their ecology and spatial dynamics, which may be reflected in their genetic structure. Besides high self-pollination, founder effects due to high metapopulation dynamics (colonization and extinction) could also create strong genetic differentiation (Ingvarsson 2002). When a new slack is colonized by seed(s) from extant populations, the new population will have a deviating genetic make-up. In addition, founder effects might also occur when vacant sites are colonized by individuals from extant populations located within the same slack. This situation is likely in very large dune slacks. Furthermore, extinction in *L. loeselii* populations, either because a slack becomes unsuitable through succession or because of stochastic events, will also increase variance among populations.

Our study revealed relatively high total genetic variation, of which 50% resided within populations. The high genetic variation found in this study is in congruent with the results of Pillon *et al.* (2007), who also observed high genetic variation in the British and French populations. Given its self-pollinating breeding system, the proportion of within-population variation in *L. loeselii* is rather high compared to other autonomous selfers (see Nybom & Bartish 2000). Relatively low levels of differentiation in highly selfing species are generally associated with high rates of seed dispersal, e.g. by means of lightweight seeds (e.g. Chauvet *et al.* 2004; Yan *et al.* 2004) or by dispersal through water (e.g. Honnay *et al.* 2010). This might be the case for *L. loeselii*, because with the assignment test we estimated a relatively high rate of gene flow in our study. Another explanation for the relatively high diversity within populations is colonization by multiple founders. Our assignment tests suggested that individuals colonizing newly available slacks came from several other slacks. This is probably the result of the fact that new slacks are formed on the beach, directly adjacent to the older slacks. Therefore, seeds that colonized the new slacks most likely come from multiple older inland populations.

We failed to detect a relationship between genetic diversity and population size and also between genetic diversity and population age. As suggested by several authors (Barrett & Husband 1997; Schmidt & Jensen 2000; Bonin *et al.* 2002), this might reflect fluctuations in population size in previous years due to unknown histories. For example, the relatively low genetic variation observed in the largest known population (Borkum 1, ~3,000 individuals) is probably due to a sharp decline in population size from a total of 11,317 individuals in 2006 to 759 in 2007 (Petersen 2010). This population bottleneck may have led to the loss of genetic diversity. It is not surprising

that the island harbouring the most populations (Texel, 8 populations) was found to have the highest genetic divesity.

In a review paper on seed dispersal, Soons (2006) concluded that the wind dispersal distance will increase with a decrease in terminal velocity and also with an increase of seed release height. With a low terminal velocity, i.e. 0.2 m/s (Tackenberg 2001) and dust-like seeds, *L. loeselii* has the potential to travel many kilometers especially under stormy conditions (Soons 2006). Nevertheless, our assignment test assigned almost all individuals to their population of sampling, indicating that most dispersal takes place within dune slacks. This is in line with other studies on terrestrial orchids that have shown that seeds mostly disperse only few meters (e.g. Machon *et al.* 2003; Chung *et al.* 2004; Jacquemyn *et al.* 2006b). These observations are also in line with the growth form of *L. loeselii*, which is much smaller than other orchids from wet dune slacks such as *Epipactis palustris* and *Dactylorhiza incarnata*. *E. palustris* for instance, has dangling fruits with longitudinal slits that probably promote wind dispersal of seeds. Furthermore, *L. loeselii* is surrounded by rather tall vegetation, in particular in older successional stages, and by high dune ridges that may limit dispersal by wind. For another dune slack pioneer species, *Parnassia palustris*, dune ridges have been suggested to form an effective barrier for dispersal (Bonte *et al.* 2012). On the other hand, dispersal over longer distances seems high enough to enable rapid colonization of newly formed dune slacks. Our assignment test on newly colonized slacks showed that most colonizers dispersed over distances between 1 to 4 km, but some colonizers had a putative dispersal distance of >100km. However, interpretation of the results of assignment tests is difficult (Paetkau *et al.* 2004). Therefore, caution must be applied when interpreting the results of long-distance dispersal, especially between islands that are hundreds of kilometers apart. Nevertheless, such dispersal events may still occur through other dispersal vectors such as bird feet or human (ecologist) boots.

Conclusion

In our studied metapopulation, *L. loeselii* showed a strong genetic structure that seems shaped mainly by its selfing breeding system. In addition, the high extinction and colonization dynamics of its temporarily available habitat might also play a role in shaping the genetic structure. Remarkable is the high genetic variation within populations, in particular for a self-pollinating species. This probably results from colonization by multiple founders. The assignment analysis demonstrated that colonization of new slacks is predominantly, albeit not exclusively, achieved from nearby populations. This implies that the number and distance of suitable habitat patches is important for the connectivity in the metapopulation. Therefore, considering the general decline of the species, conservation should focus on creating more dynamic habitat, in which new wet, calcareous dune slacks are formed regularly. With this study, we hence provide additional evidence for the importance of coastline dynamics for genetic conservation of dune slack species (Bonte *et al.* 2013, Vandepitte *et al.* 2012).

Acknowledgements

We thank State Forestry Service and Natuurmonumenten, etc. for their permission to study populations in their nature reserves, Jörg Petersen, Kees Bruin, Johan Krol, Arjan Zonderland, etc. for their information on fluctuations and long-term changes in population sizes of *L. loeselii*, Peter Kuperus and Betsy Voetdijk for technical assistance in the lab, etc. An van den Broeck (INBO) for sharing AFLP protocols and discussion of results.

Chapter 7

Summery and Synthesis

Rohani Shahrudin

In most of Western Europe, natural dune forming processes have been restricted for decades and as a consequence most dune systems are in a phase of stabilization (e.g. Provoost et al. 2011), leading to decreased biodiversity in early successional stages (Van der Maarel et al. 1985). Along the West-European coast the decline of pioneer species has become one of main concerns for nature management. Measures such as sod-removal, mowing and grazing have already started in 1950's, but at present such restoration activities are being carried out on a large scale, in particular in the Netherlands, but also in Belgium and the United Kingdom (Provoost et al. 2011). Several papers exist that have monitored and evaluated these activities on effectiveness (Grootjans et al. 2001, 2002; Van der Hagen et al. 2008). At the European level, the European country members are responsible to conserve dune slack habitats due to their high biological interest. Much research has been done to understand the causes of the species decline in wet dune slacks. For example, Sival (1997) studied effects of acidification on the decline of basiphilous dune slack species, while Lammerts (1999) studied nutrient and seed bank availability of pioneer stages. Both authors have used a chronosequence approach to study the survival of pioneer stages in a succession series. A choronosequence, in which parent material, topography, climate and many habitat factors are comparable (Harden 1982), is a very useful tool to study processes in ecosystem development over long time periods (up to a century). In this way problems of spatial variation that might lead to bias in the results, are eliminated. The present study also uses a chronosequence approach to study ecosystem development and associated changes in population structure,and genetic structure. The aim is to evaluate if nature conservation can rely on natural dune forming processes to re-initiate pioneer stages or that human intervention is needed to prolong the life span of the pioneer dune slack species.

Soil organic matter accumulation and the occurrence of alternative stable states

Soil organic matter (SOM) accumulation has been considered to be a good indicator of successional rate (Walker & Del Moral 2003, Berendse et al. 1998, see also **chapter 2** and **chapter 4**). Most wet dune slack studies have shown that the accumulation of SOM increased linearly (Sival et al. 1998; Olff et al. 1993). However, a study on SOM accumulation in Dutch dune slack with different hydrological regimes showed that SOM rates varied considerably under different hydrological conditions; slacks located in exfiltration sites had low accumulation rates, while slacks in infiltration areas had high accumulation rates (Lammerts et al. 1995). This led to our research question formulated in chapter 2: (i) which factors regulate SOM accumulation rates in wet dune slacks. (ii) how long can pioneer species, such as *Littorella uniflora,* retard the accumulation of organic matter in dune slacks?

In order to answer these questions, soils from the wet dune slacks chronosequences on the Dutch Wadden Sea islands were sampled. We found that hydrological factors, such as flooding duration, slack age, and soil pH were important, factors that influence SOM accumulation in dune slacks, but above-ground biomass was the best parameter explaining difference in SOM accumulation (**chapter 2**). The results from diachronic chronosequences, in which changes are monitored in time at the same spot, showed large differences in SOM accumulation, even within the same slack. This is in line with the results of Lammerts et al. (1995) and Adema et al. (2002), who showed that a dune slack dominated by *Littorella uniflora* remained stable for more than 80 years and SOM accumulation remained very low. They even suggested that such an ecosystem exihibited characteristics of an alternative stable state (AAS). The theory of ASS suggests that contrasting ecosystem states may co-exist under the

same environmental conditions (Beisner et al. 2003; Suding et al. 2004). *Littorella uniflora* keeps the vegetation in a stable state, probably due to it adaptations to anoxic soil conditions. *Littorella* has radial-oxygen-loss (ROL) capability; the plant is able to release large quantities of oxygen via its root system, thus stimulating nitrification in the root zone. In a system with groundwater flow, the nitrate can be transformed into N_2 by micro-organisms when the water leaves the root zone and becomes anoxic again. The N_2 then escapes to the atmosphere. This is called N-loss through coupled nitrification-denitrification (Engelaar et al. 1991, van Bodegom et al. 2005). This mechanism to reduce the availability of nitrogen, can only function when the slack has a high discharge of anoxic groundwater.

In **chapter 3**, where we tried to answer the question if alternative stable states in dune slacks really occurred, we showed that even a modest perturbation, such as a very dry period can trigger a shift from pioneer stage to a later successional stage. We concluded that given the frequent occurrences of such events, really stable states probably either do not exist, or they are exceptionally rare. In most dune slack pioneer stages are impacted by hydrological disturbances in the surrounding infiltration areas. Examples are increased evaporation by planted forests or abstraction of groundwater in the surroundings, which can easily destabilize the stable pioneer state. Therefore, we can conclude that the role of the hydrological regime is still important to sustain pioneer species. Ecosystems where ASS exists should be managed carefully. If a pioneer stage is destabilized and feedback mechanisms are active that promote a rapid accumulation of organic matter, measures such as sod cutting, mowing or grazing will not restore pioneer stages, or only for a very short time (Grootjans et al. 2002). Such (positive) feedback mechanism steer ecosystems in a direction, which may be opposite to the restoration objective (Suding et al. 2004). Although we have shown that it is quite hard to find evidence for the occurrence of ASS **(chapter 3**), we think we discovered another type of stable state in an old dune slack that was once artificially made by people. They constructed large sand dikes that prevented continued flooding of sea water, but an unforeseen side-effect was that the sand dike constructions stimulated the discharge of fresh groundwater from the surrounding areas. The re-occurring of pioneer species in the old slack of 4e Kroon's polder (Vlieland) under very high organic matter accumulation (with OM percentages > 75%) might suggest the occurrence of ASS (**chapter 2**). It started with the invasion of a hemi-parasitic species (*Pedicularis palustris*) in a dense vegetation of *Phragmites australis*. Locally, this invasion resulted in reduced vegetation cover of *Phragmites*. Hence, light availability on the surface increased considerably, allowing pioneer species to re-colonize. Even though this vegetation community differs from pioneer stages of very young slacks, it has many Red List species in common (*P. palustris* and *L. loeselii, Dacthylorhiza incarnate* and *Epipactis palustris*. If the invasion of *Pedicularis* is restricted to the current area and will not spread to neighboring sites, different vegetation communities (pioneer and later stages) will occur. Both communities will become a stable community as long as the hydrological regime remains stable (Adema et al. 2002).

These results show that not all sites are need to be managed. Managers should be aware that sometimes alternative stable states (ASS) are developing that can sustain pioneer community for a long time, without additional management.

Plant populations of pioneer stages*; resonse to environment conditions and management*

For nature managers, information on the structure of plant populations could be an effective tool to assess the effectiveness of their management strategies (Harvey 1985, Oostermeijer et al 1996, Hegland et al. 2001, Hartman & Oostermeijer 2014). Although much literature exists on this topic, population studies on plant species of pioneer stages in dune slacks are rather scarce. Therefore, two pioneer species (*Schoenus nigricans* and *Pedicularis palustris)* were selected to study their population structure in relation to environmental conditions and different management strategies (**chapter 4)**.

Our results showed that the population structure of *S. nigricans* differed between young and old populations. The population structure of young populations was dominated by seedlings, while the population structure of old populations was dominated by mature individuals. This is in line with the results of Ernst & van der Ham (1988), working in dune slacks in the Holland Coast in the Netherlands. Westhoff & Van Oosten (1991) even suggested that seed set and germination of seeds could only occur on bare soil, which would explain the dominance of mature plants in older stages of succession. Our results show that this is not an adequate explanation; we often have found juveniles of *Schoenus nigricans* in habitat wth much organic material. Ernst & Van der Ham (1988) came with a better explanation. They suggested that because *Schoenus nigricans* had ROL capability, mature plant could facilitate species that otherwise could endure prolonged inundation periods. These incoming species could restrict the survival of seedlings of *Schoenus.* Furthermore, *S. nigricans* produces much litter that also could assist other species to establish, in particular when the tussocks die.

The low abundance of seedlings in older populations of Schoenus, might also be the results of poor seed condition or poor seed set. However, our germination experiment showed that seeds from old tussocks (50 years) were even more fertile then those of younger populations. So, large tussocks in the old successional stages are important as a seed source for the population's survival, as was also pointed out by Grootjans et al. (2004) who monitored the (scarce) establishment of juveniles of *Schoenus nigricans* directly after sod cutting in a dune slack on Terschelling. In the same area, Bekker et al. (1999) analysed the soil seed bank of the vegetation in various successional stages, and found that no viable seeds were present in a the soil of a Schoenus dominated vegetation, that did had flowered abundantly during spring. This points to possible high seed predation by the Schoenus specific moth *Glyphipterix schoenicolella*, that may sometimes prevent or reduce seed set considerably (Ernst 2009).

For *P. palustris,* the population structure was not affected by mowing activity neither by slack age (**Chapter 4**). The population structure appeared to be regulated predominantly by soil wetness, which affects seedlings negatively; the number of seedlings of *Pedicularis* was particularly high at the drier sites. This finding was in accordance with the suggestion by Ter Borg (1985) that seeds of *P. palustris do* not germinate when inundated. Petrů & Lepš (2000) also observed a negative effect of flooding on germination rates of *P. sylvatica*. However, the result of our seeds sowing experiment showed low germination percentages at very dry sites. From these results we can conclude that this species has narrow tolerance for water levels during the germination stage. Even though seeds were not able to germinate in inundated site, yet they still need a certain amount of wetness to germinate (Decleer et al. 2013). A summary of the life cycle of *P. palustris* is presented in Figure 1.

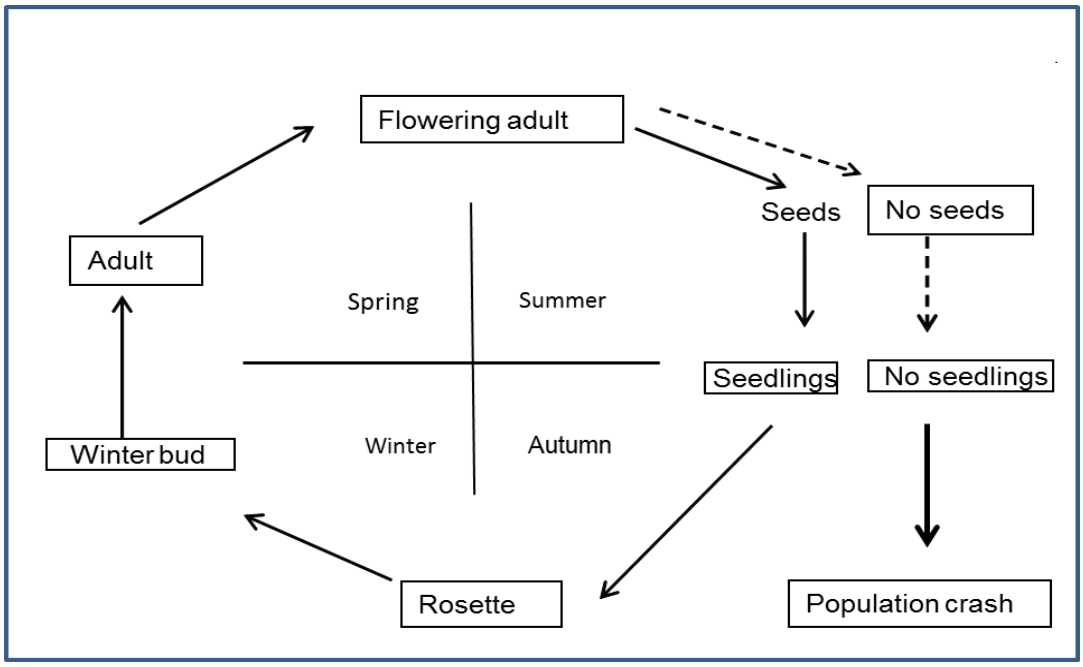

Figure 1. Biennial life-cycle of *Pedicularis palustris*. Solid arrow indicates a life-cycle during normal weather conditions, while dashed arrow indicates an extreme weather condition.

During the summer, the cycle starts when seeds are released and seedlings are established. They form a rosette in autumn, which turns into a winter bud during winter. In the next spring an adult plant emerges from the bud. Growth continues until an adult plant has developed, which bears flowers that are pollinated by bumble-bees (Kwak 1979, Ter Borg 1985). After pollination, seeds develop within seeds capsules (1300 – 1620 seeds per population; Karrenberg & Jensen 2000). Seeds then are released in summer and will germinate successfully when the soils are not inundated.

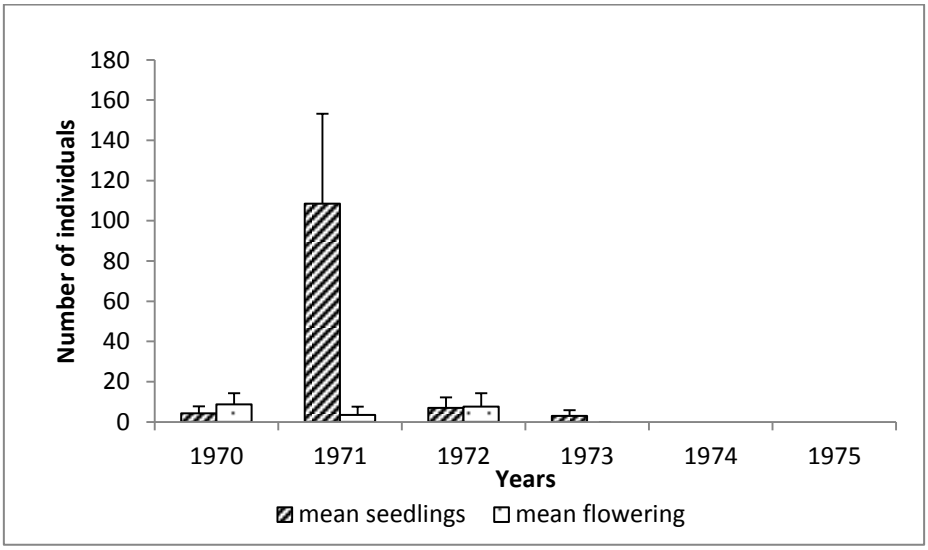

Figure 2. Graph showing number of seedlings and flowering individuals of *Pedicularis palustris* in the Kapenglop dune slack during four years of observation (1970 – 1975). (Adapted from Ter Borg unpublished data).

However, in a year with extreme weather (dry summer or prolonged winter) the population of *P. palustris* has a high chance to crash in the next year. The effect of an extreme weather condition on the populations of *P. palustris* had been illustrated by Ter Borg –unpublished data) who monitored a *Pedicularis* population for 7 years in a dune slack on the Island of Schiermonikkoog (Figure 2). The data showed that a high number of seedlings in the year 1971 did not result in a high number of adults in the next year. This was probably due to the very dry year 1971 (Grootjans et al. 1991). Thus, a dry condition seems not suitable for seeds germination as well as for the survival of seedlings.

Even though no relation was found between mowing and the population structure of *P. palustris* in the current study, however, previous studies have shown that mowing can be very beneficial for maintaining the *Pedicularis* populations as shown in the study of (Petrů & Lepš 2000, Schmidt & Jensen 2000).

Meta-population viability of *Liparis loeselii* and successional dynamics

As a protected species, the decline of *Liparis loeselii* has led researchers to identify factors that contribute to the decline and also to understand the ecology requirements for its survival. Most studies showed that the decline of the *Liparis* populations was related to the degradation of their habitat, specifically when these habitats were overgrown with vegetation that altered habitat parameters (Jones and Etherington 1992, Wheeler et al. 1998, McMaster 2001, Bednorz 2003, Bzdon and Ciosek 2006). However, there was still lack of information on how the species establishes a new population under natural condition. Besides, it was still unknown if mowing could extend the life-span of this species. To answer these questions, a study was done at the Hors area (southern tip of Texel) (**Chapter 5**). This area was one of the places where new dunes are still being formed in a natural way and the development of the dunes and dune slack is well-documented. The results showed that on Texel, the species had a relatively small window of opportunity to establish and maintain a population (~20 years). We found that mowing could extend the life-span of a population, but only 5-10 years.

Other research on the environmental conditions of *L. loeselii* populations on five Wadden Sea islands including the German island of Borkum revealed that other factors could be important as well (Figure 3). Sites (where *L. loeselii* was expected to colonize (Expected in Figure 3), were positively correlated with several parameters, which most of them indicate the influence of brackish water (EGV, Cl). Thus, this indicates that this species will not be able to colonize when the sites are still strongly influenced by salt water. Meanwhile, sites where *L. loeselii* was found in an optimal condition were positively correlated with a high pH. Extinction of *Liparis* was associated with high values of organic matter (OM) together with high values of Al and Fe).

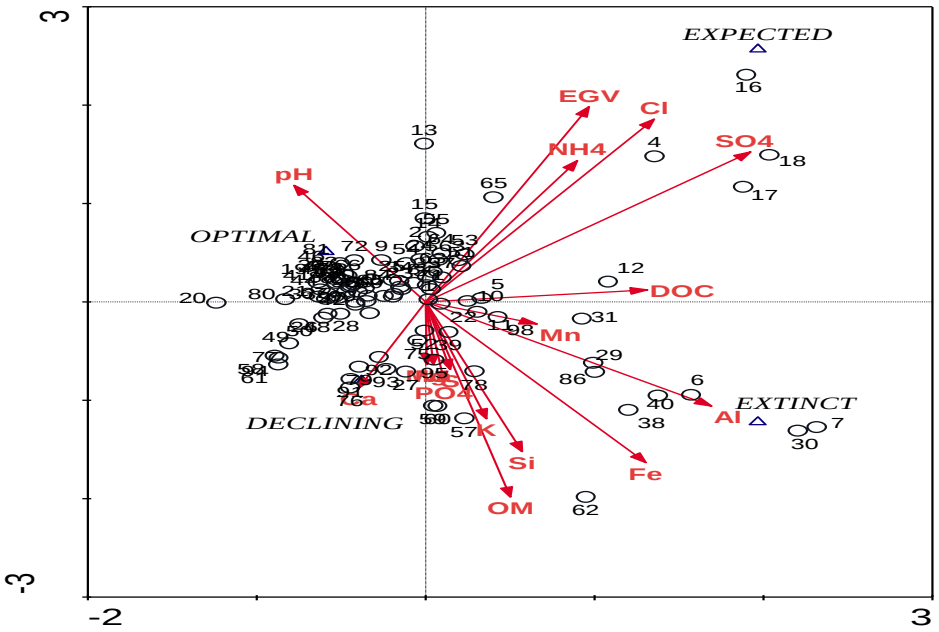

Figure 3. A CCA-biplot of site explaining the occurrence of Liparis (expected, extinct, declining, optimal) and environmental variables (Grootjans et al. 2014).

The conceptual model in Figure 4 is describing the performance of *L. loeselii's* populations throughout the successional stages. In time the population's size increases when organic matter slightly increases.

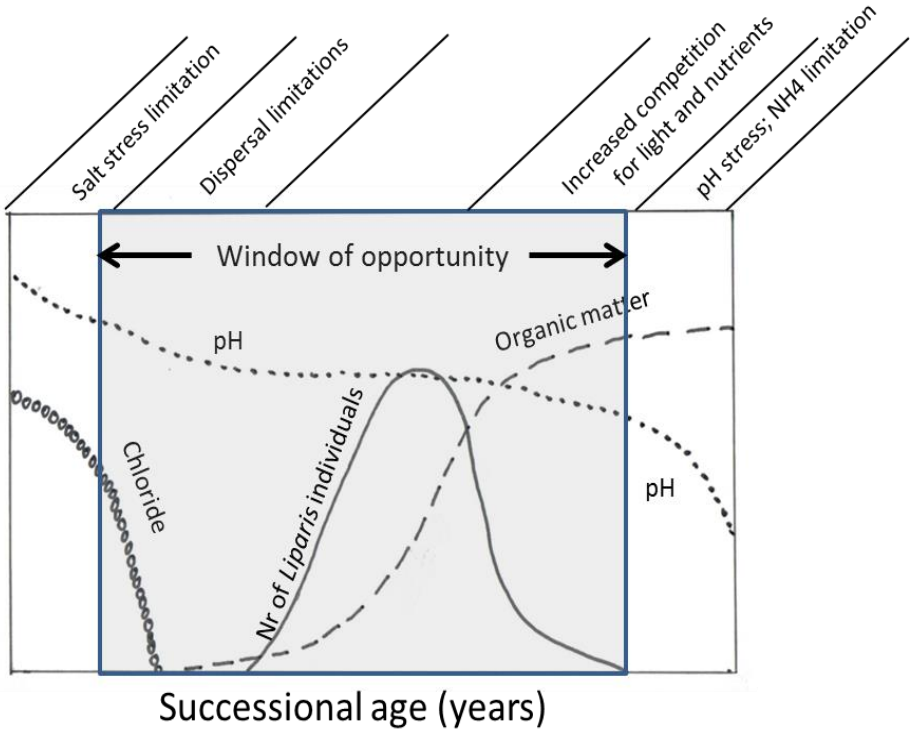

Figure 4. A conceptual model summarizing the relation of several parameters and the number of individuals of *Liparis loeselii* throughout successional age. The shaded area is the optimal condition for *Liparis loeselii*

The populations started to decline when organic matter reached a certain boundary, although the pH is still high. However, when in older successional stages the pH dropped below 6, *Liparis* populations start to decline, which is in accordance with what had indicated in previous studies (Wheeler et al. 1998, Lammerts 1999, Sival 1996).

Monitoring population sizes on the island of Borkum (Peterson 2010) showed that under natural conditions, (with a strong discharge of calcareous groundwater), but with regular flooding with sea water, but without management, populations of *Liparis* survived more than 30 years. Detailed monitoring of individuals (Figure 5) showed that between years small differences in distribution patterns could be observed. Locally individual disappeared, while also new individuals appeared in adjacent areas. From the map, we can see that in 2003 (black line), the population shifted more inward. However three years later (yellow line), the population disappeared from most of the inward site, yet expanded at the western part of the slack. In 2010, the population moved towards the sea, which means it shifts to younger sites. This process apparently can extended the population's life-span considerably, since the population as a whole on the beach plain of Borkum is already 30 old, which is longer than what have been found on Texel. This implies the importance of the formation of new habitats due to natural processes, such as the formation of new dunes (with fresh groundwater discharge) and the occurrence of regular over-wash (to keep the productivity of the vegetation low). Both are essential conditions to maintain viable populations of the orchid.

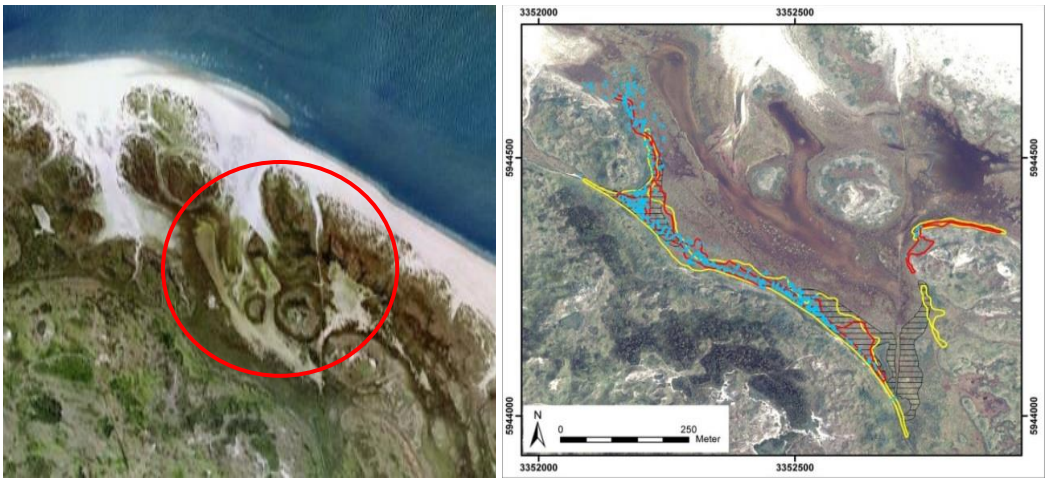

Figure 5. Beach plain area on Borkum where *Liparis loeselii* can be found abundantly (red circle) (left). The picture clearly indicates the sea water intrusion on the beach plain. This washover has affected the *L. loeselii* population (right); black line indicates the population distribution in 2003, yellow line indicates the population distribution in 2006, red line indicates the population distribution in 2009, the blue line indicates the population distribution in 2010 (source: Peterson 2011).

The network of populations reflected in the population genetic structure

Also information on the genetic variation of protected species could be useful for management startegies. For a species that inhabits a transient habitat-type like *L. loeselii* (c**hapter 5**), it is important to understand the effect of frequent extinction-colonization processes on the genetic variation of the populations. In dune slacks, the decline of habitat quality, either due to successional process or other anthropogenic factors, will lead to extinction, while the natural formation of new slacks allows the establishment of new populations through colonization. This

extinction-colonization process (also known as meta-population dynamics) was expected to decrease the genetic variation of the metapopulation due to founder effects (Pannell & Charlesworth 1999, Wang & Caballero 1999). At the same time founder effects could increase the genetic differentiation between populations. Therefore, we studied *L. loeselii* populations on six Wadden islands, in order to investigate the effect of metapopulation dynamics on the genetic diversity of the orchid (**chapter 6**). We hypothesized that a high genetic differentiation would be observed between the studied populations due to high metapopulation dynamics. We found a high genetic differentiation between the populations (F_{ST}=0.50), which is normal for self-pollinating species (Hamrick & Godt 1996). A genetic study on the same species by Pilllon et al. (2007) also resulted a strong genetic differentiation for fen and dune populations. On the other hand, a meta-analysis study conducted on the family of Orchidaceae, showed that most orchid populations have very low genetic differentiation (mean 0.146) (Phillips et al. 2012). However, this study excluded self-pollinating orchids from their study.

A separate analyses had been done to see if there are other factors influencing the genetic structure of *Liparis*. Factors that had been tested were coordinates of each populations (spatial) and environmental parameters; vegetation cover, shrubs and herbs cover, moss cover, vegetation height and organic matter depth (eco). The results showed that the variation partition of the spatial, environmental parameters and the interaction between two factors, more or less explained an equal amount of the genetic population structure of *Liparis* (Figure 6), yet all the variations were not significant. The reason why the genetic structure of the populations was less influenced by environmental parameters, might be due to the high turn-over rate of the populations (between 3 to 8 years; Hartman & Oostermeijer, 2014), which is not sufficient for local adaptation.

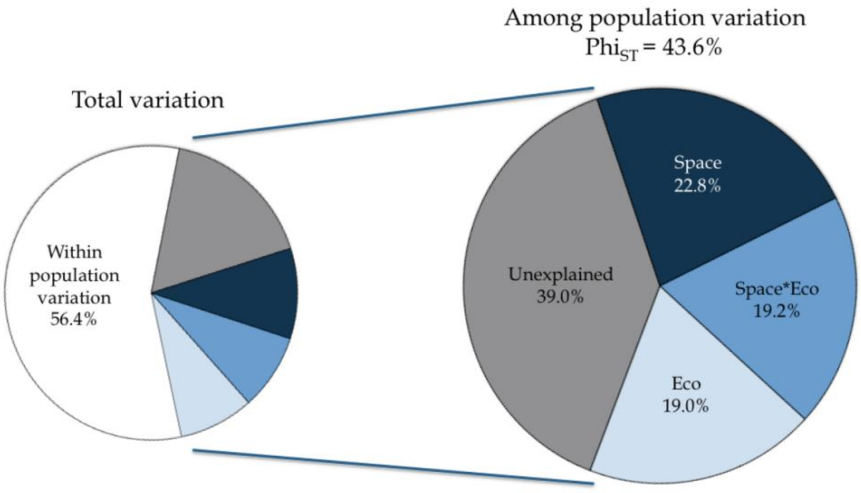

Figure 6. Variance partitioning shows the percentage of among populations genetic variation, of which the variation explained by spatial variables (Space) is 22.8%, environmental variables (Eco) is 19.0%, and interaction between the two (Space*Eco) is 19.2%. Thirty nine percent of the variation remained unexplained (adopted from van der Meer 2010)

Although we did not find a a clear isolation-by-distance pattern for the whole Wadden Sea area, on the island-level geographical distance probably did influence the genetic structure. On Schiermonnikoog, for instance, the genetic differentiation for the populations Schier 1 and Schier 5 was high, whilst the three other populations

were not genetically different (Figure 7). The populations Schier 1 and Schier 5 were located far away from each other and from the rest of the populations (Figure 8). This aspect of gene flow within an island needs further investigation.

Since *Liparis* is a self-pollinating species, we expected a low genetic variation within populations (Nybom & Bartish 2000). In contrast to our expectation, as a selfer, it was quite surprising to observe that fifty percent of the variation was found within populations (**chapter 6**). The assignment tests revealed that most of the source colonization originated from several adjacent slacks (**chapter 6**). This might suggests that the high genetic variation within populations probably originated from multiple colonization events. Even if migration between populations would occur frequently, the immigrant would not easily mix genetically with local populations (due to self-pollinating). Thus, this explained a high genetic variation within populations.

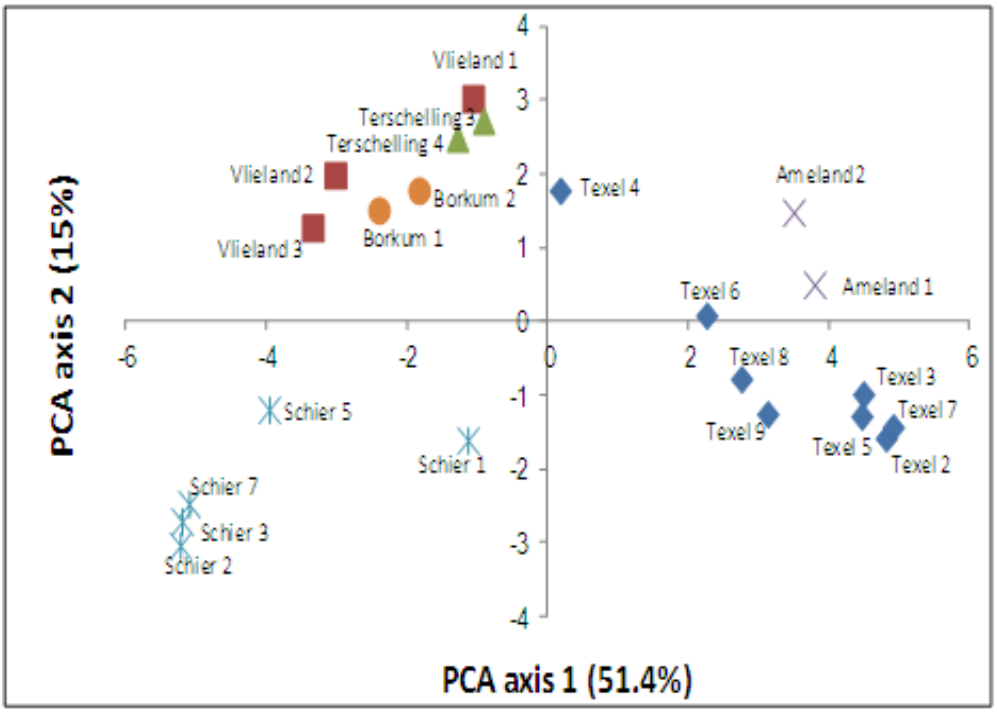

Figure 7. Principle Component Analysis of 21 populations of *L. loeselii* with population's labelling. First axis shows a high significant in genetic differentiation (F_{ST}=0.24, P=0.003), while axis 2 shows no significant difference (F_{ST}=0.065, P=0.62)

Our genetic study has improved our knowledge on the dispersal of *Liparis,* which is important for the survival of its meta-population. Despite having dust-like seeds (Arditti & Ghani 2000), most of the seeds of *Liparis* apparently do not disperse very far. Only a very small fraction of seeds disperses over longer distances, which can lead to gene flow between islands. Regular formation of new slacks may provide a temporal overlap between populations, which increases the life-span of the metapopulation. But when few new slacks are formed, overlap in time may be lacking and local extinction might occur within the metapopulation. Local extinction will lead to the loss of genetic variation, thus reducing the evolutionary potential of the species to adapt to changes in environmental conditions. Under such conditions, new introductions from population of other islands might be helpful in sustaining the metapopulation. However, the chances for such a genetic rescue from other islands are rather small because, dispersal events between islands are rare. (**chapter 6**).

Therefore, additional human interventions, such as sod removing and mowing could help the meta population viability. By doing this, succession can be delayed, thus 'buying time' for future formation of new slacks. Under the present conditions many local populations on the Wadden Sea islands may decline drastically or become extinct without human interventions.

Figure 8. The western part of the island of Schiermonnikoog, where most of the *Liparis* populations occurred. Note that populations Schier 6 and Schier 8 were not included in the genetic analysis.

The relevance of management: a conclusion

This thesis has presented information on some ecological aspects of wet dune slack vegetation, which might be useful to further develop strategies to extend the life-span of rare pioneer species. Our studies have also shown that not every slack requires management. Management does not seem to be necessary when environmental conditions keep the pioneer vegetation in a stable state. This thesis has reported several (relatively) stable states in dune slack succession on the Wadden Sea islands. Therefore, it will be very efficient if we can detect the occurrence of alternative stable states in dune slack ecosystems before management measures are being executed. Although the ideal approach for sustaining pioneer species in dune slacks is encouraging new slack formation, this is only possible in certain areas, outside urban areas and without risk for coastal defense. Under such conditions regular management, as mowing and sud cutting is still needed to ensure the survival of many local population within the metapopulation of the island.

Samenvatting

Rohani Shahrudin

Duinvalleien zijn de natte componenten van een duinsysteem. Ze zijn doorgaans niet permanent nat en behoren dus tot de categorie “seasonal wetlands”. Met name in de jonge vegetatiestadia van kalkrijke duinvalleien op de Waddeneilanden komen nogal wat soorten voor die in de rest van Europa beperkt zijn tot laagvenen (“fens”). Of liever, die soorten kwámen voor, want in veengebieden van NW-Europa zijn ze extreem zeldzaam geworden, door ontwatering en eutrofiering van die venen. In zekere zin zijn de jonge ontwikkelingsstadia in kalkrijke duinvalleien dus de laatste bolwerken waar veel ‘laagveensoorten’ nog standhouden. Maar ook die standplaatsen worden bedreigd door natuurlijke successie (vegetatieontwikkeling), en ook door menselijke beïnvloeding van die successie, door bijvoorbeeld, luchtverontreiniging, ontwatering en aanplant van (dennen)bossen. Als de successie te snel gaat door menselijke beïnvloeding hebben veel zeldzame soorten ook een geringe kans op overleving. Om die snelle successie tegen te gaan worden al jaren beheersmaatregelen uitgevoerd, zoals maaien, begrazen of – nog rigoureuzer – plaggen, waarbij de hele organische toplaag wordt verwijderd om de successie opnieuw te laten beginnen.

Dit proefschrift gaat over de variatie die ontstaat door het uitvoeren van beheersmaatregelen, maar ook over wat er gebeurt wanneer natuurlijke processen ervoor zorgen dat er nieuwe groeimogelijkheden ontstaan voor bedreigde plantensoorten (**Hoofdstuk 1**). Door deze vergelijking kunnen we uitspraken doen over de effectiviteit van de beheersmaatregelen.

Hoofdstuk 2 gaat over de rol van organische stof bij de vegetatieontwikkeling van pionier stadium naar eindstadium in duinvalleien. De snelheid van organische stofophoping is in duinvalleien op de Waddeneilanden gemeten en op twee manieren vergeleken. De ene manier is om gebieden van verschillende leeftijd ruimtelijk te vergelijken (“synchronic chronosequence” benadering). De andere manier is om veranderingen in de tijd te volgen en wel op dezelfde plek (“diachronic chronosequence” benadering). De eerste benadering hebben we toegepast op twee eilanden (Schiermonnikoog en Terschelling). De ontwikkeling van de organische stof in de tijd was heel vergelijkbaar, alleen was de hoeveelheid organische stof in het eindstadium op Schiermonnikoog veel hoger dan op Terschelling.

De tweede, veel betrouwbaardere, benadering liet zien dat er grote verschillen bestaan tussen valleien in de snelheid van organische stofophoping. Die verschillen werden bepaald door de hydrologische omstandigheden, maar vooral door de verschillen in bovengrondse biomassa.

Valleien met een laagproductieve vegetatie, zoals van Oeverkruid (*Littorella uniflora)*, stapelden vrijwel geen organische stof in de bodem, ook niet na meer dan 50 jaar. Oeverkruid is een laagproductieve soort en houdt de voor hem gunstige situatie in stand door zuurstof in de bodem te lekken (radial-oxygen-loss; ROL). Door die zuurstof breekt zijn eigen dode materiaal snel af. Door die zuurstof wordt er in de bodem ook ammonium omgezet in nitraat. Het door Oeverkruid niet gebruikte nitraat stroomt voorbij de wortels en als de bodem verder zuurstofloos blijft zetten bacteriën de nitraat om in gasvormige stikstof die naar de atmosfeer ontwijkt. Op die manier voorkomt Oeverkruid dat hoogproductieve soorten gebruik kunnen maken van deze stikstof in de bodem en voorkomt daarmee dat ze zich uitbreiden. Daarmee wordt ook de successie vrijwel stilgezet. Duinvalleien met soorten die geen ROL mogelijkheden hebben begonnen veel eerder met het stapelen van organische stof in de bodem en het ging ook veel sneller naar een hoogproductief eindstadium, zelf wanneer er beheersmaatregelen werden toegepast om de successie te vertragen.

Een sterk vertraagde successie gedurende soms 90 jaar wijst op het bestaan van een zgn. “Alternative Stable State” (AAS). In **Hoofdstuk 3** wordt onderzocht of een dergelijke AAS ook werkelijk stabiel is in duinvalleien. Hiervoor werden in de Koegelwieck op Terschelling zgn permanente kwadraten gebruikt waarin de vegetatiesamenstelling gedurende bijna 20 jaar op dezelfde plek beschreven werd, alsmede enkele abiotische factoren, zoals pH en organische stof in de bodem. Een analyse van de verandering in soortensamenstelling van de vegetatie liet zien dat, hoewel de soortensamenstelling van plekken met verschillende leeftijden lang bleef verschillen, uiteindelijk toch bijna alle plekken op elkaar gingen lijken. Dus de verschillen zijn in de Koegelwieck uiteindelijk niet stabiel gebleken.

In **Hoofdstuk** 4 worden effecten beschreven van milieufactoren en beheersmaatregelen op populaties van Knopbies (*Schoenus nigricans)* en Moeraskartelblad (*Pedicularis palustris)*. Deze soorten zijn kenmerkend voor soortenrijke stadia van duinvalleivegetaties en worden vaak bij elkaar aangetroffen. Van de populaties werden in het onderzoek drie levensstadia beschouwd; zaailingen, juvenielen (jonge planten die nog niet bloeien) en volwassen planten (die bloeien en zaden maken). De zaden van volwassen planten werden getest op vitaliteit door ze in het laboratorium te laten kiemen onder optimale omstandigheden. Een deel van die zaden werd in een veldexperiment gebruikt om te testen onder welke omstandigheden in het veld de zaden konden kiemen.
Hoewel Knopbies en Moeraskartelblad in hetzelfde milieutype voorkomen blijken de milieufactoren toch een verschillende invloed te hebben op de populatieopbouw van de soorten. De populatieopbouw van Knopbies werd eigenlijk alleen door de factor tijd beinvloed, terwijl overstromingstijd voor Moeraskartelblad heel bepalend was voor de leeftijdsopbouw van de populatie. Knopbiespopulaties lijken kalkrijke omstandigheden bij lage organische-stofgehalten te prefereren, terwijl populaties van Moeraskartelblad voorkomen bij een wat bredere range in gemeten milieufactoren. Maar uit een veldexperiment bleek dat deze soort wat betreft kieming heel gevoelig is voor (te) hoge, maar ook voor te lage waterstanden.

De Hoofdstukken 5 en **6** gaan over Groenknolorchispopulaties in relatief snel veranderende duinvalleien. Deze streng beschermde orchidee gaat in Europe met name achteruit in veengebieden, waarbij veranderingen in de veenbodem door bijvoorbeeld ontwatering nagenoeg onomkeerbaar zijn. In duinvalleien kan de successie echter vrij eenvoudig door plaggen weer opnieuw beginnen als het milieu voor Groenknolorchis niet meer geschikt is en ook door nieuwvorming van duinvalleien krijgen lokale populaties van Groenknolorchis steeds opnieuw een kans om te overleven. **Hoofdstukken 5** beschrijft onder welke omstandigheden Groenknolorchis zich vestigt en weer verdwijnt in een serie min of meer natuurlijke duinvalleien op Texel. Een populatie van Groenknolorchis wordt in het onderzochte gebied ongeveer 20 jaar oud; door het instellen van een maairegime kan de levensduur met nog ongeveer 5 jaar verlengd worden. Bij toenemende organische stofgehaltes in de bodem en bij een dalende pH gaat de soort achteruit.

In **Hoofdstuk** 6 wordt de genetische structuur van de metapopulatie van de Waddeneilanden onderzocht. Er bleek een hoge differentiatie te bestaan tussen verschillende populaties van Groenknolorchis. Dit komt waarschijnlijk door het regelmatig uitsterven en weer opnieuw vestigen van lokale populaties in duinvalleien (hoge metapopulatiedynamiek). Een onverwachte uitkomst van onze studie was dat ondanks het feit dat Groenknolorchis hele kleine zaden heeft, ze waarschijnlijk toch niet grotendeels over zeer grote afstanden door de wind wordt verspreid. De meerderheid van nieuw gevestigde populaties was afkomstig uit de nabije omgeving,

en slechts een heel klein deel van de nieuwe populaties was het resultaat van verspreiding over hele grote afstanden (van eiland naar eiland). Ons onderzoek naar de opkomst en neergang van Groenknolorchis populaties op de Waddeneilanden laat zien hoe belangrijk het is voor de overleving van de soort om steeds nieuwe jonge duinvalleien te laten ontstaan, bij voorkeur door natuurlijke processen, maar als het niet anders kan ook door beheersmaatregelen.

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The process of searching potential supervisor was quite interesting – sending e-mails to some potential supervisors from all over the world, and finally it worked only with one researcher whose name is Albert Pieter Grootjans (he asked me to called him Ab) from Groningen, the Netherlands. I arrived in the Netherlands on a cold winter morning in February 2009. My first experience was totally opposite to my anticipation. Having spent almost 2.5 hours in the train from Schiphol to Groningen, I wondered; why is this land so flat? When I arrived in Groningen, I was welcomed by Ab, Baps – his wife, and Bikila – another PhD student, at the train platform. It was a warm welcoming session at a very cold train station. Ab brought me to the Zernike campus (mind you that it was Sunday), showed me my office, coffee corner and briefed me on other technical issues. From that day, my PhD journey had begun under his supervision. Ab, you have teached me a lot and never let me down. You are not only a promotor and supervisor, but you are more than a student could asked for. At the beginning I was a little bit worried when you supervised me closely. Later on, I understood, that is was because I did not have a strong background in the field, you had to 'hold my hand' and walk together with me. Then you released my hand slowly, you let me walk alone, even though I kept shouting for help. But you were always there, ready to give your hand without hesitation. Your patience, understanding and tolerance when guiding your students, your passion and enthusiasm in research, leave a great impact on me and my carrier. I will not forget my first (and probably the last) experience when we hitch-hiked back from the German island of Borkum, because no busses were provided. To Baps, thank you very much on your effort to prepare a nice dinner for me. I know it was not an easy task to make sure the meals would meet my requirements. And it was even harder for you to not offer anything to me during the last party since I was fasting. I felt like I was at home when we had dinner together like a family, and thanks for a nice chit-chat before we went to bed. I

want to express my thanks to my co-promotors, Gerard Oostermeijer and Annemieke Kooijman. Gerard, thanks for your willingness to accept me to work with you, which enabled me to work in Amsterdam, and make it my second home (after Groningen). You encouraged and challenged me to think further and to see my research in broader sense. Even though it always took some time to get your comments on my manuscript, but it was worthwhile. Your comments always led to another perspective that I never thought of. I was also impressed with your photography skills and thanks for the picture of *Liparis loeselii* that you gave to me. Working with 2 gentlemen, it is always nice to work with a lady. Annemieke, you inspired me to be a good field biologist. I learnt from you on how to sample in a systematic way, and to take notes during meetings. I was honored to share a room with you when we both participated in the IMCG field excursion and conference in Slovakia and Poland.

When most people wish to go to the Wadden Sea islands for vacation, I am among those who are so fortunate to go to the islands almost every week during the summer. It is such a privilege to work in the nature areas of these islands. This only possible with the help of people that are managing these areas. I thank them not only for the permission, but also for the information and facilities they offered. Firstly I would like to thank Otto Overdijk (Natuurmonumnenten, Schiermonnikoog), for being such a nice and easy-going manager. I'm sure you will be remembered by many students that work on the island for your true kindness and your enthusiasm in conserving nature. Many thanks to Henk Everts, for his willingness to travel and work with me on Schiermonnikoog. To Wim Penning, thanks for your information on the vegetation on Schiermonnikoog. I had a great time with you in the field, discussing vegetation and nature, and also about the people on the island. To Jitske Esselaar, thanks for giving the permission to work on Texel. Thanks to Kees Bruin, for providing information about *Liparis loeselii* on Texel and for showing the hidden spot of the orchid. Many thanks to Arjan Zonderland, who showed me *Liparis* sites, and allowed me to stay in the Staatbosbeheer house on Terschelling. On Vlieland, I owe Evert Jan Lammerts a lot, for his help in the Kroon's Polders; thank you! Finally, I want to convey my appreciation to Jörg Petersen who assisting me during work on Borkum and helped to get the information about *Liparis* on that island.

Back to the office environment, I start with Groningen - I was first welcomed and introduced to Annemiek, the secretary. Annemiek, you are the most efficient

secretary that I ever met. Many thanks for your help in claiming travel expenses, since I did lots of “binnenland en buitenland” travels, and many other administration-related stuff. Also thanks for the extra information on the beauty advices, and for encouraging me to do exercises. Eventually, I fell in love with body-fit, which later turned out to be negatively correlated to my stress levels. My letter of acceptance was signed by Ton Schoot Uiterkamp, the head department during that time. Ton, I always enjoyed discussing with you. I admire your genuine passion in research and people. Thanks for introducing me to Foreign Guest Club (now known as WIRE), which gave me the chance to meet more people that shared the same problem - living abroad in a completely different world. Our worries and confusion later on, were shared by the WIRE committee, consisting of people that had much experience in living outside their country. Then we knew that everything was all right. To Marianne Uiterkamp, thanks for sharing your experiences. I liked the discussion on the similarity and differences of cultures and traditions between regions. Also thanks for the tips on relationships and also on the tips of how to find Mr. Right. That one was a very handy tips for me.

To our head department; Henk Moll, thanks for your help on administration and financial matters. It was a great relief to know that I was able to get reimbursement from the department for my travel expenses. My working experience at IVEM was great with a great people around me, thanks to the IVEM’s members. To a regular coffee corner and lunch members; Sanderine, thanks for inviting me to your house and for the excursion on the dairy farm and also for offering the perfect place for the IVEM’s international food party. I appreciate the time you provided to listen to my ambitions, my swimming progress, and to share other random life matters. Rene, I am already missing your lunch-calls, and thanks for lending me the shoes for my swimming test. Your effort in explaining to me the Dutch conversations was most appreciated, otherwise I would not have a clue. Michiel, sorry for not being able to speak Dutch, but at least I can understand the conversations a little bit, and you

know that I am not really bad at it right? Karin, working with you during the international food party and Christmas breakfast was really fun, thanks for included me in those events. Rachel, thanks for the bubble wrap that you gave to me. Gideon, you are a foreign student information ‘counter’. Your creativity, your well-armed current information, tricks and tactics helped us a lot. Thanks for fixing my bike’s chain. I learnt from you about the architecture of a bike and I will always remember your transformer bike. Jan Hessels, I have to confess that in the beginning it was

rather difficult to engage with you. But later on, we get along fine and it was fun to greet ‘hoi’ to you. I really appreciated that you picked me up when I about to ‘sink’ in my office, and chat with me, it was comforting. Maria Jose, thanks for your care and concern. You comforted me when I really needed it during hard times. And to Julio, thanks for sacrificing your weekend, rode the bakfiets back and forth while transporting my stuff. Federico will grow up as the luckiest kid in the world having parents like you. To Reino and Ron, we knew each other only for a few months, but it was great to know both of you. To my officemate, Bikila, thanks for being an understanding roommate and for being a good listener. Sorry because I was not able to help much during your difficult time, but trust me, you and your family were always in my prayers.

Amsterdam opened a new chapter in my life and it was a colorful experience. First, I thank Pieter van Tinderen for accepting me in his group. Thanks to Eric Schranz for assisting me during the early days, to Yorike for sharing your experience in working with *Liparis*, to Melis and Hoe for your time discussing about PhD life and about experiments. A special thanks to Thomas Van Hengstum, for helping me to carry my belonging from Amsterdam to Groningen. Sorry for the stomach-ache trouble, until now I do not have any clue why my stomach always gives problem when I move. To Patrick Meirmans, thanks for your patience, I believe it is difficult to work with a person without sufficient knowledge on genetics like me. But I was honored to work with a great geneticist like you. The lab works cannot be completed without help from lab technicians. Thanks to them, especially to Peter Kuperus, for being patience with

me and with all the problems with AFLP, you are really a great lab technician. Also my thanks to Betsy Voetdijk, for all your assistances, and to Lin Dong, thanks for inviting me for a weekend break at your house. To Louis Lee, it was great that I could speak Bahasa Melayu, which I was longing for when I missed my country, terima kasih! To other members; Rob, Ludek, and Harold, it was so fun to have lunch together followed by coffee-after-lunch session, you guys rock!

My work benefitted greatly from the work of several master students. I would like to thank Sascha van der Meer for the collaboration in the genetic study on *Liparis loeselii*. It was such an expedition to collect the *Liparis* samples, starting with lots of confusion when trying to obtain permits. We supported each other during a tedious and tiring lab works, especially when the PCR-products did not work out. We shared

jokes and sometimes we had arguments, but that did not affect our relation. Annelies van der Craats also contributed much to the population work on *Liparis* on Texel. Annelies, thanks for your parents car that enabled us to travel to Texel for the first time and also for the nice moments that we shared during sampling. To other students; Willmer Woudwijk, Pieter van de Hoop and Coen Verboom, thanks!

My life abroad could be hard without family and friends. I want to thank Fatma Zaki, the first person that I contacted to get information about life in Groningen. A cheerful and positive person, your concern and understanding touched me. I thank you for the time you spent with me, listening to my problems, even in your busy day, jazakallah! Then, I was introduced to Azzahrawani and Yusof, the only Malaysian student in Groningen during that time. Thanks for everything, I am indebted a lot to both of you. I would say without this couple, I might quit to live in the foreign land. They never complaint, and they have taught me the beauty side of hardship. To Dyah Ekashanti Oktarina, thanks for accommodating me during your busy days, and to let me putting my things in your studio when I had to move to Amsterdam. The moment that I will not forget was when you were willing to share my sadness and providing a shoulder to cry on. To Nishath Hamza, thanks for accommodating and cook for me during Ramadan. Your perseverance in facing the hard tribulations during your PhD was inspiring to me. To Nicola Cho and Kim Gargar, thanks for our friendship. We met in the Dutch class and became good friends. It seems that we grew up together in Groningen. From Kim, I learnt to be a cheerful and positive person. As I am writing this, Kim is still detained, I hope that he will be released soon and finish his study. Then we can proceed with our plan to collaborate in botanical research for our region. Nicola, I think you know me almost in-and-out, since you were the first person that I contacted if something happened. Thanks for being such a wonderful and understanding friend. I miss to chat with you, and really hope to see you again in the future. To Ee Soo Lee, thanks for being a great travel-buddy and a great friend. Travelling with you was so much fun, even without a proper plan. Simple things can be an exciting subject for us. I thanks Ziad for his help and information that he provided. He introduced me to the DeGromiest, which enabled me to make more friends. To the DeGromiest members, thanks for inviting me to your monthly gathering and weekly Quran recitation. To Aizati and Amir, thanks for being such understanding and helpful friends. To Dayah-Leiden, thanks for your help when I needed it badly and for your companion during various trips. To other Malaysian

friends; Kak Ra, Iza, Warga-Wage, Rotterdammers (including Uncle Joe) and many more, thanks for the information, the accommodation when I needed to be in Schiphol early in the morning, the support and the prayers.

In Groningen, I met with lots of wonderful people. I was grateful to be invited in ‘the ladies group”. Hanke, from the first time I met you, I knew that you are a strong lady. Your positive attitude towards life and your faith were the characters that I admired most in you. Other sisters; Habon, Aletta, Daisy, Jolien, Selma, Satia, Heleen and Yusra, thanks for inviting and accepting me in the circle. Julie Haris, it was so nice to know you. Thanks for all the help and care that you gave to us, the Malaysian community in Groningen. I am so lucky to have family in Groningen. To tante Alief, thanks for being like a mother to me. Together with other family members – Gulbahar, Mehmet, Turhan, Sevda, Aihan and Burhan, which made my attachment with Groningen even stronger. I hope you will once be repaid for all your kindness and for the shelter you provided for me during the first and final weeks in Groningen. However, not only shelter, but also love and care and not to forget the scrumptious Turkish cuisine.

To the supporters in Malaysia, firstly to the members of Biological Department (JASBIO), my sincere gratitude especially to my head of the department - Dr. Faridah, to the ex-bosses; Dr Aziz and Dr Mariam, to my mentor – Dr Jamilah, to seniors and friends, thanks to all of you. To my ‘me-ngetips’ friends, thanks for your support, mentally and spiritually. You guys never failed in believing in my ability. These are friends that one couldn’t ask more. Finally to my siblings; Osman, Zaid,

Norhayati and Hamdi, and my parents; Shahrudin and Jarizah, thank you very much for the unconditional love and for your prayers.

“*We all travel for different reasons, and taking off on a solo trip seems to be a very powerful experience that will change not only how you view travel, but also impact who you are as a person*" - Anonymous

Curriculum Vitae

Rohani Shahrudin was born on 25th October 1980 in Perlis, Malaysia, where she grew up and attended primary and secondary schools until 1997. After finishing her pre-university level there, she moved to Selangor to do a Bachelor Degree in Biology at the Universiti Putra Malaysia in 1999. The program has exposed her with lots of fieldworks in tropical forests, which triggered her interest in conservation studies. For her final year project, she studied genetic techniques to conserve a wild orchid, *Paphiopedilum* spp. under the supervision of Dr. Faridah Qamaruz-Zaman. After finished her first degree in 2003, she accepted a job as an operation officer in a wholesale company. Realizing of her own potential, Rohani decided to quit her job in 2004 and continued her study at Master-level at the Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Under the supervision of Dr. Mohd. Nizam Mohd. Said and Dr. Wan Juliana Wan Ahmad, she assessed the community structure and floristic variation pattern of trees in the Kenong Forest Park, Malaysia. After completed her thesis, she joined the Universiti Teknologi Mara as a temporary lecturer teaching Botany. In 2007, she was appointed as tutor at the Universiti Malaysia Terengganu, which enabled her to apply for a full scholarship to study abroad. In February 2009, she started her PhD. research on coastal wetland plant ecology at the University of Groningen, the Netherlands, under the supervision of Prof. A.P.Grootjans. Rohani is now resuming her career as a lecturer at the School of Marine Science and Environment, University Malaysia Terengganu. Her present research is concentrated on conserving wetland ecosystems, with emphasize on coastal wetlands.

Publications

Peer-reviewed publications:

Shahrudin, R.B., Woudwijk, W., de Hoop, P., Dullo, B.W. & Kooijman, A.M., Grootjans, A.P. 2014. Accumulation rates of organic matter in wet dune slacks**; i**nfluence of water level regime and plant productivity. *Plant & Soil.* Accepted.

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Nizam, MS, Juliana, WA, Raffae, A, **Rohani, S** and Sani, M (2005) Community Structure of Tree Species at the Sg. Bebar Peat Swamp Forest, Pekan, Pahang. PSF Technical Series No. 4. UNDP/GEF Peat Swamp Forest ProjectNizam, MS, **Rohani, S,** and Wan Juliana, WA (2012) *Floristic variation of tree communities in two distinct habitats within a Forest Park in Pahang, Peninsular Malaysia. Sains Malaysiana*.

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